

Award Number: W81XWH-12-2-0076

**TITLE:** Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections

**PRINCIPAL INVESTIGATOR:** David S. Perlin, Ph.D.

**CONTRACTING ORGANIZATION:** Rutgers, The State University of New Jersey  
Newark, NJ 07103-2757

**REPORT DATE:** October 2015

**TYPE OF REPORT:** Annual

**PREPARED FOR:** U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

**DISTRIBUTION STATEMENT:** Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

# REPORT DOCUMENTATION PAGE

*Form Approved  
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

<b>1. REPORT DATE</b> October 2015	<b>2. REPORT TYPE</b> Annual	<b>3. DATES COVERED</b> 30 Sep 2014 to 29 Sep 2015		
<b>4. TITLE AND SUBTITLE</b>  <b>Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections</b>		<b>5a. CONTRACT NUMBER</b> W81XWH-12-2-0076		
		<b>5b. GRANT NUMBER</b>		
		<b>5c. PROGRAM ELEMENT NUMBER</b>		
<b>6. AUTHOR(S)</b> David S. Perlin, Ph.D.  E-Mail: perlinds@njms.rutgers.edu		<b>5d. PROJECT NUMBER</b>		
		<b>5e. TASK NUMBER</b>		
		<b>5f. WORK UNIT NUMBER</b>		
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b>  Rutgers, The State University of New Jersey Newark, New Jersey 07103		<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>		
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		<b>10. SPONSOR/MONITOR'S ACRONYM(S)</b>		
		<b>11. SPONSOR/MONITOR'S REPORT NUMBER(S)</b>		
<b>12. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				
<b>13. SUPPLEMENTARY NOTES</b>				
<b>14. ABSTRACT</b> In the third year, the effect of pH changes on the bactericidal behavior of CHD-FA was assessed <i>in vitro</i> against medically important bacteria. The potent bactericidal activity of CHD-FA was significantly diminished and became bacteriostatic only when the pH was increased from 1.7 to 7.0. Upon establishing the burn wounds, the <i>in vivo</i> efficacy of CHD-FA to treat burn wounds infected with multidrug resistant Gram-negative bacteria <i>K. pneumoniae</i> or Gram-positive bacteria MRSA was evaluated. In all these trials, histopathological analyses and wound healing gene expression profiling demonstrated alleviated inflammation and promoted healing upon CHD-FA treatment, however no significant bacterial burden reduction was observed from CHD-FA treated wounds. Thereafter, different formulations of CHD-FA were assessed for improved <i>in vivo</i> antimicrobial potency on both cutaneous and burn wounds. CHD-FA zinc diacetate (CHD-FA-Zn) was found the most promising. In the MRSA infection study, bacteria were completely eliminated from infected wounds as rapidly as 24h post-inoculation upon CHD-FA-Zn application. Promising <i>in vivo</i> antimicrobial efficacy was also observed in the short-course <i>P. aeruginosa</i> study. However, the microbial burdens restored rapidly from day 3 in the 6-day study. The biofilm matrix formed on day 3 post-inoculation may be restricting the penetration of the drug. During the next quarter and throughout this next year, we will perform studies with a modified application method (dressing) to enhance absorption of drugs through the burn wound eschar and the biofilm matrix. Overall, we have made significant progress in the third year in demonstrating the value of CHD-FA to treat wound infections. We believe that the CHD-FA-Zn combined with the modified application method will be effective in treating wounds infected with major multi drug resistant pathogens and demonstrate its universality for preventing wound infections and promote healing.				
<b>15. SUBJECT TERMS</b> carbohydrate-derived fulvic acid (CHD-FA), drug resistant wound infections,				
<b>16. SECURITY CLASSIFICATION OF:</b>		<b>17. LIMITATION OF ABSTRACT</b>  UU	<b>18. NUMBER OF PAGES</b>  54	<b>19a. NAME OF RESPONSIBLE PERSON</b> USAMRMC
<b>a. REPORT</b> U	<b>b. ABSTRACT</b> U	<b>c. THIS PAGE</b> U		<b>19b. TELEPHONE NUMBER</b> ( <i>include area code</i> )

## Table of Contents

	<u>Page</u>
<b>Introduction.....</b>	<b>4</b>
<b>Body.....</b>	<b>5</b>
<b>Key Research Accomplishments.....</b>	<b>47</b>
<b>Reportable Outcomes.....</b>	<b>48</b>
<b>Conclusion.....</b>	<b>49</b>
<b>References.....</b>	<b>50</b>
<b>Appendices.....</b>	<b>51</b>

## **Introduction**

The objective of this study is to demonstrate the potent antimicrobial properties of carbohydrate-derived fulvic acid (CHD-FA) against a broad collection of drug-sensitive and multi-drug-resistant (MDR) pathogens commonly associated with wound infections, and assess the relative efficacy of CHD-FA against induced wound infections in *in vivo* animal models. Overall, this work is intended to establish CHD-FA as a safe and effective agent that can be deployed to prevent the onset of drug-resistant bacterial and fungal infections in military and civilian personnel suffering traumatic wound infections. Given its novel mechanism of action and preliminary activity against MDR bacteria and antifungal-resistant fungi, the early use of CHD-FA is advantageous because it represents a novel target that will not select for resistant organisms, and prevents the use of more specific but more limited spectrum antibiotics. The overall goal of this preclinical program is to establish a firm justification for progressing to human trials to determine the efficacy of topical CHD-FA in preventing wound infections in injured military and civilian personnel.

## **Body**

### **Description of Overall Progress in the first Annual report. (Sept 2012 to Sept 2013).**

#### **Section 2.1 Description of Overall Progress**

As described in the Annual Technical Report submitted on 10/29/2013, Specific Aim 1 of the statement of work establishing the *in vitro* efficacy of CHD-FA against drug resistant bacteria and fungi was completed. In the first year of this research contract we demonstrated the antimicrobial properties of Carbohydrate-Derived Fulvic Acid (CHD-FA) against a broad collection of multi-drug resistant bacterial and fungal pathogens commonly associated with wound infections. We completed the objectives in specific aim 1 of the award statement of work by determining the *in vitro* susceptibility of CHD-FA against a collection of multi-drug resistant *Acinetobacter baumanii*, *Pseudomonas aeruginosa*, carbapenamase-resistant *Klebsiella pneumoniae*, *Enterococcus faecium*, *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, Enterobacter spp., other clinically important species as well as azole resistant fungal pathogens *Candida* spp., *Aspergillus fumigatus* and polyene-resistant non-fumigatus *Aspergillus* species, *Fusarium* species and Zygomycetes. Most bacteria show complete growth inhibition at 0.06 – 0.125% fulvic acid, while efficacy with fungi is 0.125-0.5%. As stated in specific aim 2, small animal models were started during this period. We have also established a cutaneous wound infection model in rats with MRSA and *Pseudomonas aeruginosa* to assess (CHD-FA) as a potent topical agent to prevent drug resistant wound infections and promote healing as part of specific aim 2 of the research contract. Daily topical applications of CHD-FA were placed on the cutaneous wounds in rats at 4 and 24h post infection for up to 10 days. Wound size and wound bacterial burdens were measured to assess the treatment efficacy of CHD-FA. CHD-FA treated animals had improved wound healing and reduced bacterial burden.

#### **Section 2.2. Description of Overall Progress during the second Annual report. (Sept 2013 to Sept 2014).**

As described in the Annual Technical Report submitted on 10/29/2014, in the second year, a cutaneous wound model in rats with the drug resistant Gram negative bacteria *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and pathogenic mold *Aspergillus fumigatus* was established. Efficacy of CHD-FA against cutaneous wounds infected with these organisms was assessed respectively. Significant wound surface area reduction with high and mid CHD-FA treatment doses was observed with all studied infection models. Remarkable bioburden reduction induced by CHD-FA was also observed in wounds infected with multidrug resistant *E. coli* and *K. pneumoniae*. To better assess wound healing upon CHD-FA treatment at cellular and molecular level, a more comprehensive histopathological evaluation and expression profiling of wound healing genes was performed at various time points during the infection/treatment course in rats infected with MRSA and *P. aeruginosa*, respectively. Histopathological analysis showed that the infection and inflammation in the wounds from both infection models treated with CHD-FA was better controlled relative to the untreated and antibiotic control groups, as indicated by lower neutrophils scores as early as day 3. The decrease in cellular inflammation and increase in epithelialization at the endpoint of the experiment suggested wound healing was significantly improved with CHD-FA treatment. Host gene expression profiling displayed the same dynamic trend of differentially expressed wound healing genes upon CHD-FA treatment in both infection models. The levels of overexpression were found to be more prominent in the CHD-FA group at day 3 compared to the untreated control, and their expression levels rapidly returned towards baseline at day 6, as the same set of genes in the untreated control increased. In particular, the key biomarker of impaired wound healing IL6 was constantly overexpressed day 3 to day 6 in the untreated wound. In contrast, expression of IL6 was significantly dampened at day 6 in the CHD-FA treated wounds, indicating an accelerated and better

controlled wound healing. A full preliminary evaluation of CHD-FA to treat wound infections with all the pathogens listed in the Statement of Work (SOW) has now been completed with the submission of this annual report. These preliminary results are encouraging and histopathological and host gene expression profiling on wounds infected will be performed with the other pathogens listed in the statement of work. Other wound infection models such as the burn model and deep tissue (thigh) model will also be performed in the final year.

### **Description of Overall Progress during the current Annual Report. (Sept 2014 to Sept 2015).**

During the last 3 quarters, we continued working on specific aim 2 of the statement of work and established burn wound model in rats with the drug resistant Gram negative bacteria *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, and Gram positive Methicillin Resistant *Staphylococcus aureus*. We assessed the treatment efficacy of a newly formulated Carbohydrate-Derived Fulvic Acid combined with zinc acetate (CHD-FA-Zn) against cutaneous and burn wounds infected with these organisms.

### **Section 3. pH effects on the Antimicrobial Susceptibility testing of CHD-FA.**

A modified standardized broth-based *in vitro* susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” was used to determine the CHD-FA MIC values at a pH range of 3.0 to 7.0 for *Pseudomonas aeruginosa* –Xen05.

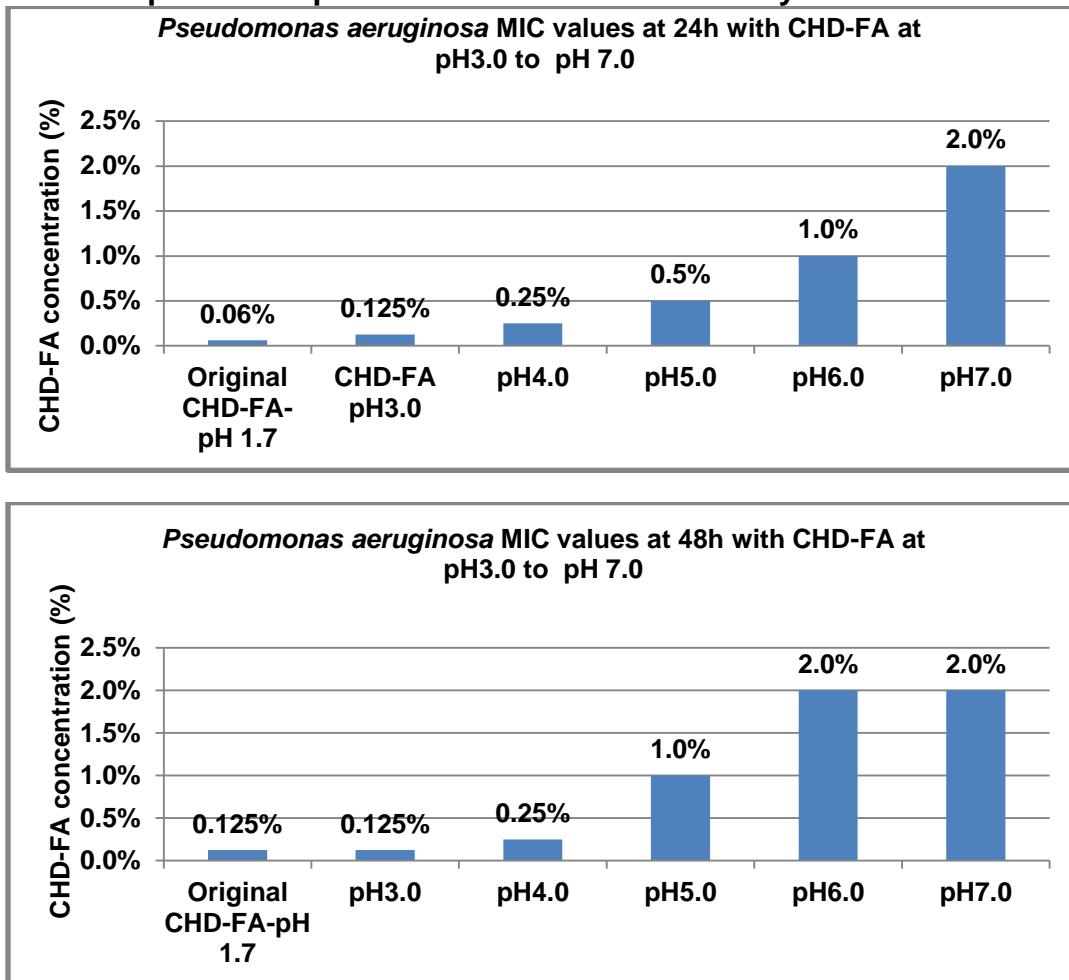
CHD-FA was supplied as a 4.6% solution by Fulvimed (Pty) Ltd (A division of Fulhold Ltd.), Somerset West, South Africa. CHD-FA was stored in the dark at room temperature. The batch number used was P191/12. The pH of the original CHD-FA was 1.7. The pH of CHD-FA was adjusted at a range from pH 3.0 to pH7.0. Half milliliter aliquots of original CHD-FA solutions were used to adjust the pH using sterile 5M NaOH. This pH modified CHD-FA solutions were used to determine the MIC values at different pH for *P. aeruginosa*, to assess MIC differences between CHD-FA at a pH range of 3.0 to 7.0. The MIC is defined as the lowest concentration producing a prominent decrease in turbidity compared to the drug free well. All assays were performed in duplicate.

The CHD-FA MIC at its original pH of 1.7 is 0.06% at 24h and 0.125% at 48h. The MIC of CHD-FA was highly dependent on pH as MIC values increased 4 fold with a shift in pH from 1.7 to 4.0. (See **Table 1** and **Figure1**). At pH 7.0, 16 fold higher concentration of CHD-FA was needed to fully inhibit the growth of *P. aeruginosa*.

**Table 1. MIC of *Pseudomonas aeruginosa* (Xen05) to CHD-FA between pH3.0 to pH7.0**

<b>Pseudomonas aero. -Xen5</b>	<b>MIC →</b>	<b>24hr</b>	<b>48hr</b>
Original CHD-FA-4.6% Stock soln pH1.7		0.06%	0.125%
CHD-FA-4.6% Stock soln -pH3.0		0.125%	0.125%
CHD-FA-4.6% Stock soln -pH4.0		0.25%	0.25%
CHD-FA-4.6% Stock soln -pH5.0		0.5%	1.0%
CHD-FA-4.6% Stock soln -pH6.0		1.0%	2.0%
CHD-FA-4.6% Stock soln -pH7.0		2.0%	2.0%

**Figure 1. Relationship between pH and CHD-FA *in vitro* efficacy at 24h and 48h**



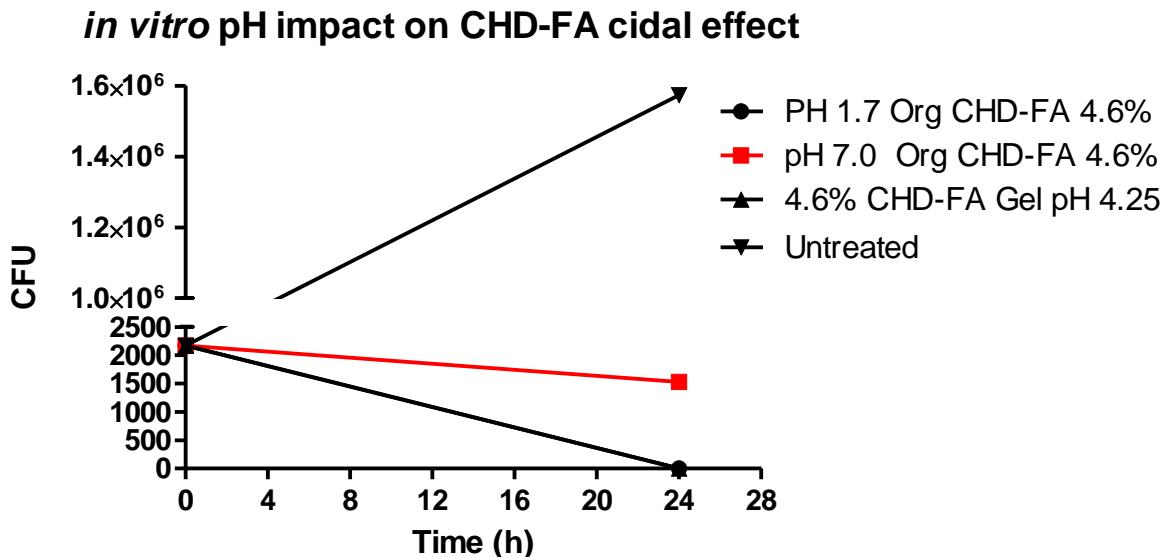
#### Section 4. Changes in pH affect bactericidal properties of CHD-FA.

The bactericidal effect of CHD-FA was evaluated as result of the high MIC shift observed due to pH increase. *Staphylococcus aureus* – MRSA strain ATCC 29213 was used for these experiments. A spectrophotometer was used to prepare the bacterial inoculum to a 0.5 McFarland turbidity standard or approximately  $1.0 \times 10^8$  CFU/ml similar to the antimicrobial susceptibility testing as described above. The cells were serially diluted to a concentration of  $1.0 \times 10^4$  CFU/ml in 2x Mueller Hinton broth and 200µl of cell suspension containing 2000 CFU bacterial cells were mixed with equal volume of the drug solution including: 4.6% CHD-FA solution at pH 1.7, 4.6% CHD-FA solution at adjusted pH 7.0 and 4.6% CHD-FA gel at pH 4.25. Untreated control was tested in parallel. All the samples were incubated at 37°C for 24h, then 50µl of each sample was plated on LB plates in duplicate for viable counts. As shown in **Figure 2**, the original CHD-FA solution at pH 1.7 and gel formulation at pH 4.25 completely wiped out bacterial cells within 24h. However, at pH 7.0 the CHD-FA lost bactericidal efficacy, instead, displayed more bacteriostatic properties as the bacteria population kept steady with only slight reduction compared to the starting inoculum. Normal proliferation was observed with untreated MRSA bacteria.

#### Conclusion:

The bactericidal properties of CHD-FA are dependent on pH. The pH of CHD-FA may be altered in wounds and may require modification to its formulation to maintain low pH in wound sites to improve wound healing and prevent infection.

**Figure 2. *In vitro* pH impact on bactericidal properties of CHD-FA**

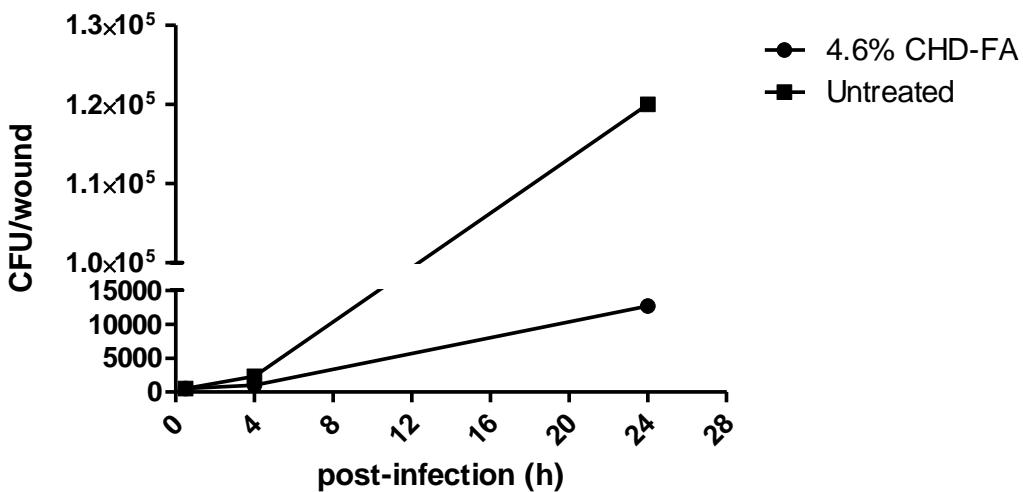


**Section 5. Evaluation of CHD-FA to treat cutaneous wounds infected with low dose MRSA in 24hr study**

A low dose MRSA cutaneous wound infection was performed to address the microbial burden persistency observed in the high dose ( $10^7$  CFU) MRSA infection model with various CHD-FA treatment groups. To ensure reproducible microbial burden recovery the experimental endpoint was limited to 24h of infection to minimize host clearance of bacteria. Five rats were in two cohorts: treated and untreated (sham) controls at 30min, 4h and 24h post-infection time points. MRSA Xen31 strain was prepared as previously described (2<sup>nd</sup> Annual Technical Report 10.2014). Wounds were created and infected with 50  $\mu$ l of MRSA cell suspension to a final infection dose of 500 CFU. Rats were given treatments of 4.6% CHD-FA, in 25  $\mu$ l volumes starting at 30min post infection. The 30min treatment starting time was used to determine if significant improvement in burden reduction would be observed.

No significant change in microbial burden was observed at 4h CHD-FA treatment time point. However at 24h post infection ~1 log burden reduction for the CHD-FA treated wounds, relative to the untreated controls was observed. (**Figure 3**).

**Figure 3. The 24h Microbial burden in low dose MRSA cutaneous wounds**



## Conclusion

The reduction of the bacterial burden in the wound sites at 24h with the lower MRSA infection dose demonstrate the potency of CHD-FA to treat wound infections.

### Section 6. Expression profiling of host wound healing genes in cutaneous wounds infected with MRSA strain ATCC 29213

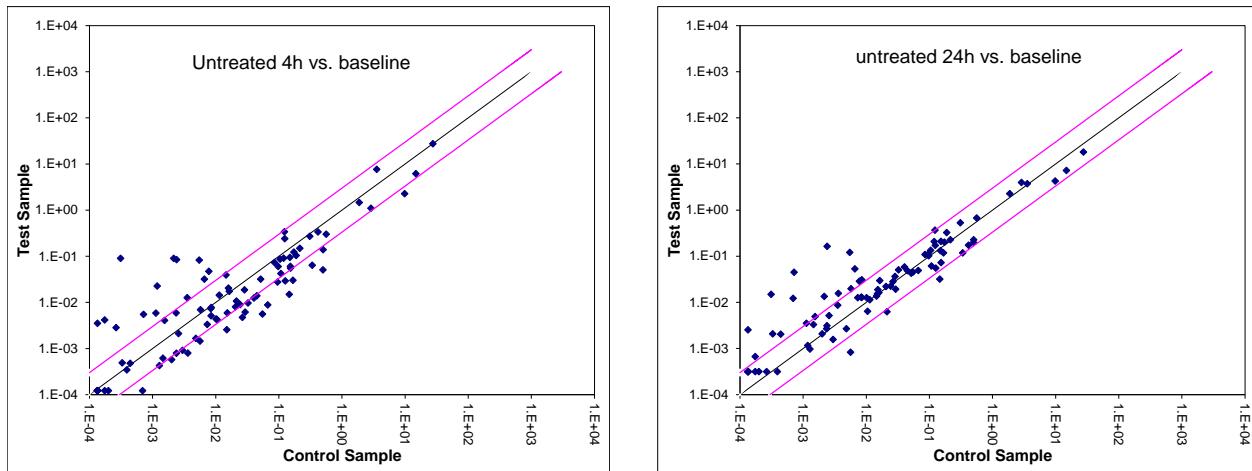
Host gene expression profiling was performed to better assess the molecular mechanisms of wound healing with CHD-FA treatment in the early stages of infection against cutaneous wounds infected with MRSA. Wound healing progresses via three overlapping phases: inflammation, granulation and tissue remodeling. Tissue healing and remodeling profiling was evaluated using a commercial PCR array (The Rat Wound Healing RT<sup>2</sup> Profiler™ PCR Array, Cat No. 330231, Qiagen). This kit assayed 84 genes essential to wound healing process, including extracellular matrix (ECM) remodeling factors, inflammatory cytokines and chemokines, as well as growth factors and major signaling molecules.

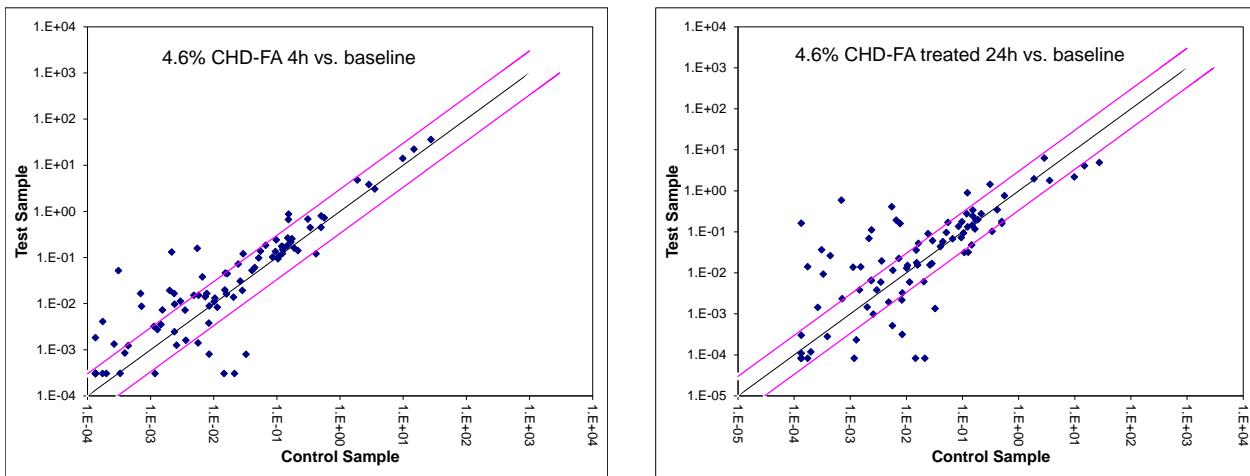
Expression profiles of wound healing genes were compared between samples treated with CHD-FA and vehicle control at 4h and 24h post infection. Consistent with results reported previously (2nd Annual Technical Report 10.2014), we observed a panel of genes with increased expression including CCL12, CCL7, CSF2, CSF3, CXCL1, CXCL11, CXCL3, CXCL5, IL10, IL1b, IL6, MMP9, PTGS2, SERPINE1, and TNF; and decreased expression were found with genes ACTC1, CDH1, Col14a, ITGB6, and TGFA, from samples collected at both time points. At 4h post-infection time point, expression changes were similar in CHD-FA treated and untreated wound (**Figure 4a**).

However, at 24h post-infection, CHD-FA treated wound displayed more pronounced expression changes compared to the untreated wound (**Figure 4a**). Of particular, inflammatory chemokine CXCL3 and CXCL5, and anti-inflammatory factor IL10 were expressed at much higher level in the CHD-FA treated wound than in the untreated wound at 24h post-infection, although the expression levels of these genes were very close in wounds from both treatment at 4h post-infection (**Figure 4b**). In addition to the well-known anti-inflammatory nature of IL10 (1, 2), CXCL3 and CXCL5 have also been reported to induce endothelial cell proliferation *in vitro* and angiogenesis *in vivo* (1, 3).

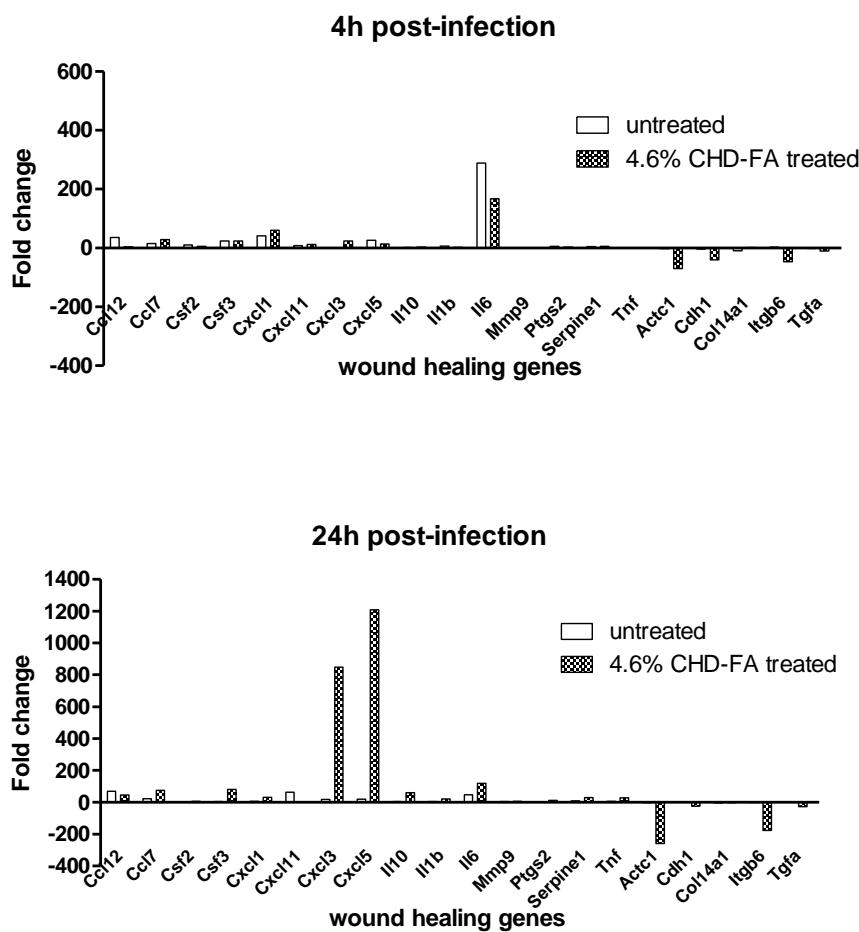
Therefore, the superimposed effect of greater overexpression of these genes in the CHD-FA treated wound relative to the untreated control is indicating more rapid and balanced inflammation response and progression toward accelerated wound healing in the CHD-FA treated wounds.

**Figure 4a. Scatter plot expression profiling of host wound healing genes.**





**Figure 4b. Fold changes of host wound healing genes treated with CHD-FA.**

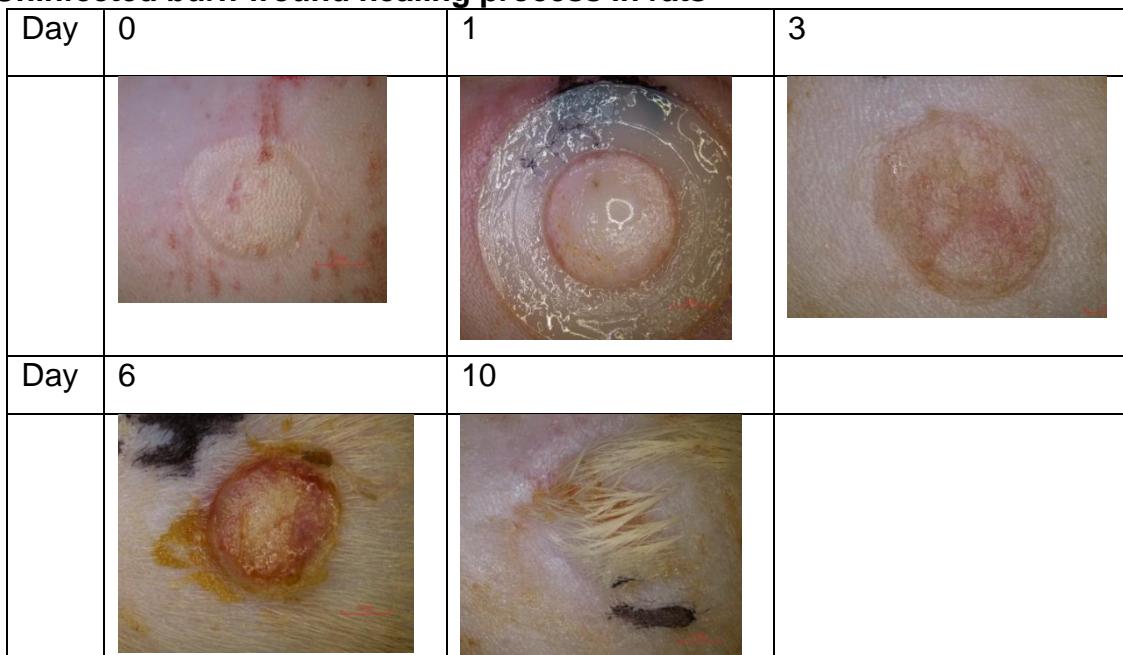


## Section 7. Establishing the burn wound model in rats.

The purpose of this pilot trial was to observe natural wound healing process and to establish the kinetics of such process in rats without infection by digital measurement of wound (burn eschar) size over time. Four Male Sprague Dawley rats (~200g) were anesthetized by intraperitoneal injection of 100mg/kg ketamine +10mg/kg xylazine. The dorsal side of the rats were shaved with electrical clippers and chemically depilated. The exposed skin was wiped with betadine. Two symmetrical burn wounds were created on the dorsum of each rat using a heated 1 cm cylinder steel block for 10 seconds, providing ~10% total body surface area for the burn wounds. Sterile polyurethane rings were placed over the fresh wounds. The rings were attached with surgical adhesive. Wounds were

covered with a Tegaderm visible adhesive dressing, and rats were rehydrated with saline administered via subcutaneous injection. The analgesic buprenorphine slow release (0.05mg/kg) was administered every 3 days to minimize pain during recovery. Daily digital wound measurement were taken with a Nikon 4x Stereomicroscope with FS-1 digital camera. Wound closure as percentage of wound surface area from days 1 through 10 was measured. Wound healing was observed for 10 days, during which Tegaderm dressings were changed daily. On Day 2, Day 3 and Day 6 one rat was euthanized to collect tissue samples for histological evaluation on the uninfected wounds as previously described in quarterly report Q6.04.2014. **Figure 5** below shows representative images of the wounds over the course 10 days. The burn eschar is discolored and blistered when created. Clear discharge is observed in some of the burn eschars until day 2. On day 3 new skin is observed under the eschar. On days 4 through 6 the burn eschar continues to fall off and replaced with regenerated skin. By day 10 the regenerated skin has normal activity with the presence of fur. Wound measurement in this model was not practical as the burn eschar did not shrink and was only shed during the course of healing.

**Figure 5. Uninfected burn wound healing process in rats**



## **Section 8. Establishing the burn wound infection with Methicillin Resistant *Staphylococcus aureus* (MRSA).**

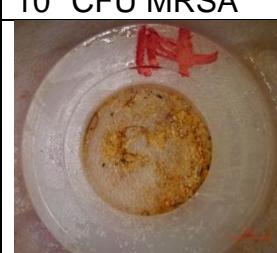
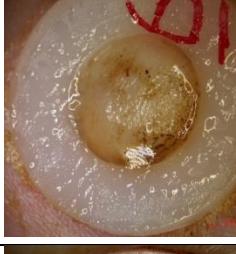
Ten rats were randomized into three infection groups (3 rats per group) and one uninfected control. Methicillin Resistant *S. aureus* strain Xen31 was inoculated in Brain Heart Infusion (BHI) media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to infect the burn wounds at  $1 \times 10^9$ ,  $1 \times 10^8$ , and  $1 \times 10^7$  colony forming units (CFU) per 0.025 ml for the infection. Two burn wounds were created on each rat, for a total of eighteen wounds. The process of anesthetizing the rats and creating the wounds was the same as described above for the burn wound model. After wound creation, rats from each challenge group were infected with 0.025 ml of the MRSA cell suspension with corresponding doses via injection to the burn site. The final infection doses for the rats were  $1 \times 10^9$ ,  $1 \times 10^8$ , and  $1 \times 10^7$  CFU, respectively. **Figure 6** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 10. Purulent discharge was observed from wound in all infection doses in the early stages of infection (up to day 3). Biofilm formation under the burn eschar was observed by day 3 in all infection doses and was consistent among the cohorts. No morbidity or mortality was observed in the rats

during the experimental period, indicating the high infection dose of this MRSA strain is well tolerated by the rats. Wound measurement in this infection model was not practical as the burn eschar did not shrink due to biofilm formation from MRSA overgrowth at the wound site.

#### **Conclusion:**

All three infection doses of MRSA ( $10^7$ ,  $10^8$  &  $10^9$  CFU) produced a non-lethal infection. The burn eschars from all 3 doses had highly purulent discharge early in infection (up to day 3) and developed a heavy biofilm till the experimental end point (day 10). The uninfected burn eschar did shed by day 6 and skin regeneration was observed. The non-lethal high bacterial infection dose would not be practical for efficacy study due to a rapid biofilm formation in addition to being a very unrealistic infection scenario. Based on these results a lower dose burn wound model study with Methicillin Resistant *S. aureus* (MRSA) strain Xen31 was used in follow-up experiments to assess CHD-FA efficacy.

**Figure. 6. Burn wound in rats with MRSA infection**

Day	Rat #1 $10^9$ CFU MRSA	Rat #6 $10^8$ CFU MRSA	Rat #7 $10^7$ CFU MRSA	Rat #10 Uninfected
0				
1				
3				
6				
10				

## **Section 9. Evaluation of CHD-FA to treat burn wounds infected with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain Xen31**

To assess the efficacy of CHD-FA against burn wounds infected with MRSA, 21 rats were randomized into four groups: 6 in 4.6% CHD-FA group, 6 in 0.46% CHD-FA group, 6 in vehicle control group, and 3 in mupirocin control group. MRSA strain Xen31 was inoculated in BHI media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $2 \times 10^5$  CFU per ml for the infection. Two burn wounds were created on each rat, for a total of 42 wounds. The process of anesthetizing the rats and creating the wounds was the same as described above for the burn wound model. After wound creation, rats from each challenge group were infected with 0.05 ml of the MRSA cell suspension with final infection doses at  $1 \times 10^4$  CFU/wound via intradermal injection to the burn site. Rats were given daily treatments of 0.05 ml of 4.6% CHD-FA, 0.46% CHD-FA, or vehicle control, or appropriate amount of 2% mupirocin cream, starting at 30 min post inoculation. **Figure 7** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 6. Purulent discharge was observed from wounds in all treatment groups in the early stages of infection (up to day 3). Wound measurement in this infection model was not a practically useful metric for treatment efficacy as the burn eschar did not shrink due to biofilm formation from MRSA overgrowth at the wound site. Purulent discharge was observed in all rats, however the duration and amount of discharge in the CHD-FA treated rats at 4.6% were less than that in the untreated controls on days 1 to 3. The bacterial burden assessment of the wounds at varying time points was shown on **Table 2**. Consistent with observations reported previously, no significant burden reduction was observed from CHD-FA treated wounds compared to the vehicle control treated wounds. Nevertheless, the mupirocin topical cream sterilized the infected wounds in 6 days.

Histopathological evaluation of wounds from each treatment group was also performed throughout the study period at days 1, 3, and 6 post-inoculation. Due to the different nature of cutaneous and burn wounds, the scoring criteria was modified (**Table 3**) to best fit the burn wound healing analysis. Compared to the vehicle control, both doses of CHD-FA treated wounds had lower neutrophils scores at day 1, followed by the peak at day 3 and dropped at day 6 (**Figure 8**). A significant lower number of neutrophils in the 4.6% CHD-FA treated wounds were observed at day 6 compared to day 3, and the score was lower than all other treatment groups, indicating prominently alleviated inflammation upon CHD-FA treatment. Another inflammation indicator, macrophages score, was lower in the 4.6% CHD-FA treated wound than the vehicle control from day 3 till the study endpoint day 6, in line with the observation on neutrophils scoring. In addition, epithelialization in wounds treated with CHD-FA was improved than the untreated (vehicle) wounds, demonstrating a more advanced wound healing. No significant difference on scores for the presence of fibroblasts and neovascularization between CHD-FA treated and untreated wounds in this trial.

Wound healing gene expression profiling was also performed using a commercial PCR array (The Rat Wound Healing RT<sup>2</sup> Profiler™ PCR Array, Cat No. 330231, Qiagen), as previously described. Similar with what observed from the cutaneous wounds infected with MRSA, a panel of genes including CCL12, CCL7, CSF3, CXCL1, CXCL11, CXCL3, CXCL5, IL10, IL1b, IL6, MMP9, PLAUR, PTGS2, SERPINE1, and TIMP1 were significantly up-regulated post infection (**Figure 9a**). The kinetic changes of these genes during the study period were compared among treatment groups (**Figure 9b**). At day 1 post inoculation, expression profiles were similar in CHD-FA treated and untreated wound, with extremely highly expressed inflammatory chemokines, especially CXCL3 (**Table 4**),

indicating a provoked inflammation on the wounds upon infection. With the time progressing to day 3, up-regulation of these inflammatory chemokines was dampened more pronouncedly in wounds treated with CHD-FA than that treated with vehicle control (**Table 4, Figure 9b**). The faster restoration of the up-regulated inflammatory gene expression in CHD-FA treated wounds once again demonstrated the potent anti-inflammatory property of CHD-FA in this wound model. Moreover, the key biomarker of impaired wound healing, IL6, restored toward baseline expression level faster in the CHD-FA treated wounds by day 3, compared to the vehicle treated control, manifesting accelerated and better controlled wound healing upon CHD-FA treatment.

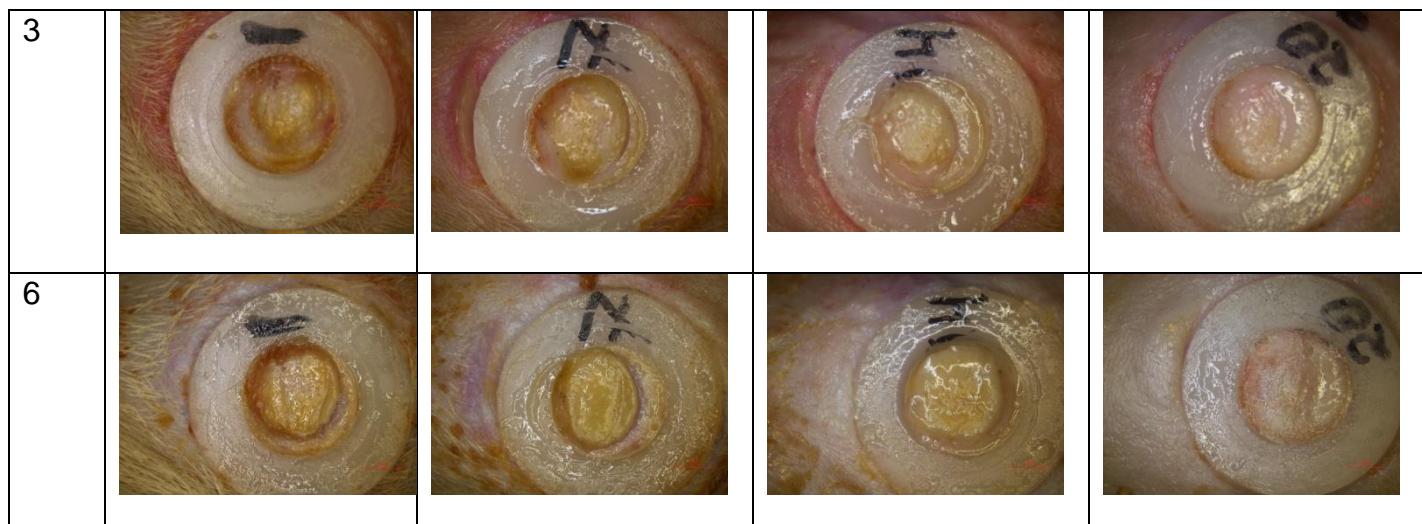
Interestingly, burn wounds treated with mupirocin demonstrated a delayed inflammatory response pattern, which showed little or no expression changes from baseline levels for both CXCL3 (inflammation) and IL6 (wound healing impairment) at day 1 but a greater level of changes were observed for day 3 and day 6 time point samples. A plausible explanation is that rapid microbial clearance from the antibiotic against the low dose infection suppressed host inflammation response at the beginning of treatment. However, while wound healing progressed, a more prominent inflammatory, anti-inflammatory interaction emerged.

## Conclusion

CHD-FA treated burn wounds infected with low dose MRSA did improve the wound healing by histopathological and host wound healing gene profiling analyses. No significant changes in microbial burden reduction were observed in this trial as observed previously in the cutaneous wound infection model. The bactericidal activity of CHD-FA may be affected by drug penetration through the eschar or high pH levels under the eschar.

**Figure 7. Burn wound images of rats infected with  $10^4$  CFU MRSA (Xen31) and treated with CHD-FA at 0.5h post infection.**

Day	Rat # 1 4.6% CHD-FA	Rat # 7 0.46% CHD-FA	Rat # 14 Vehicle	Rat # 20 AB* Treated
0				
1				



\*2% Mupirocin

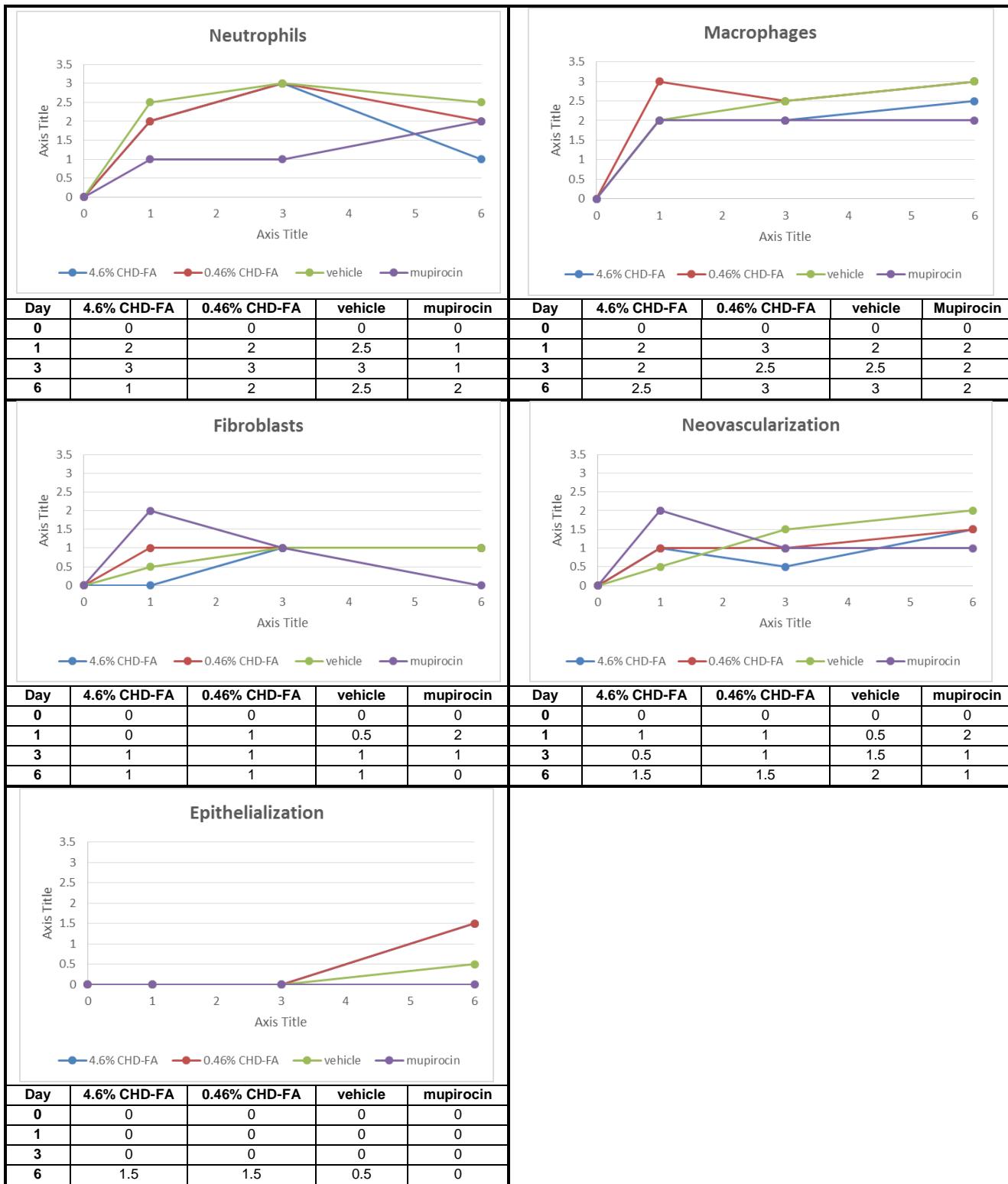
**Table 2. Bacterial burdens of burn wounds infected with MRSA at Day 1, and Day 3, Day 6 experimental endpoints.**

MRSA $10^4$ CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	6.3	NA	NA	8.2	8.1-8.4	NA	6.9	6.3-7.5	NA	MRSA $10^4$ CFU 0.5h Tx
4.60%	6.5	NA	-0.2	8.1	7.8-8.3	0.2	7.5	NA	-0.6	
0.46%	6.4	NA	-0.1	8.2	NA	0.0	7.0	6.8-7.2	-0.1	
AB Control	4.3	NA	2.0	5.6	NA	2.6	sterilized	NA	NA	

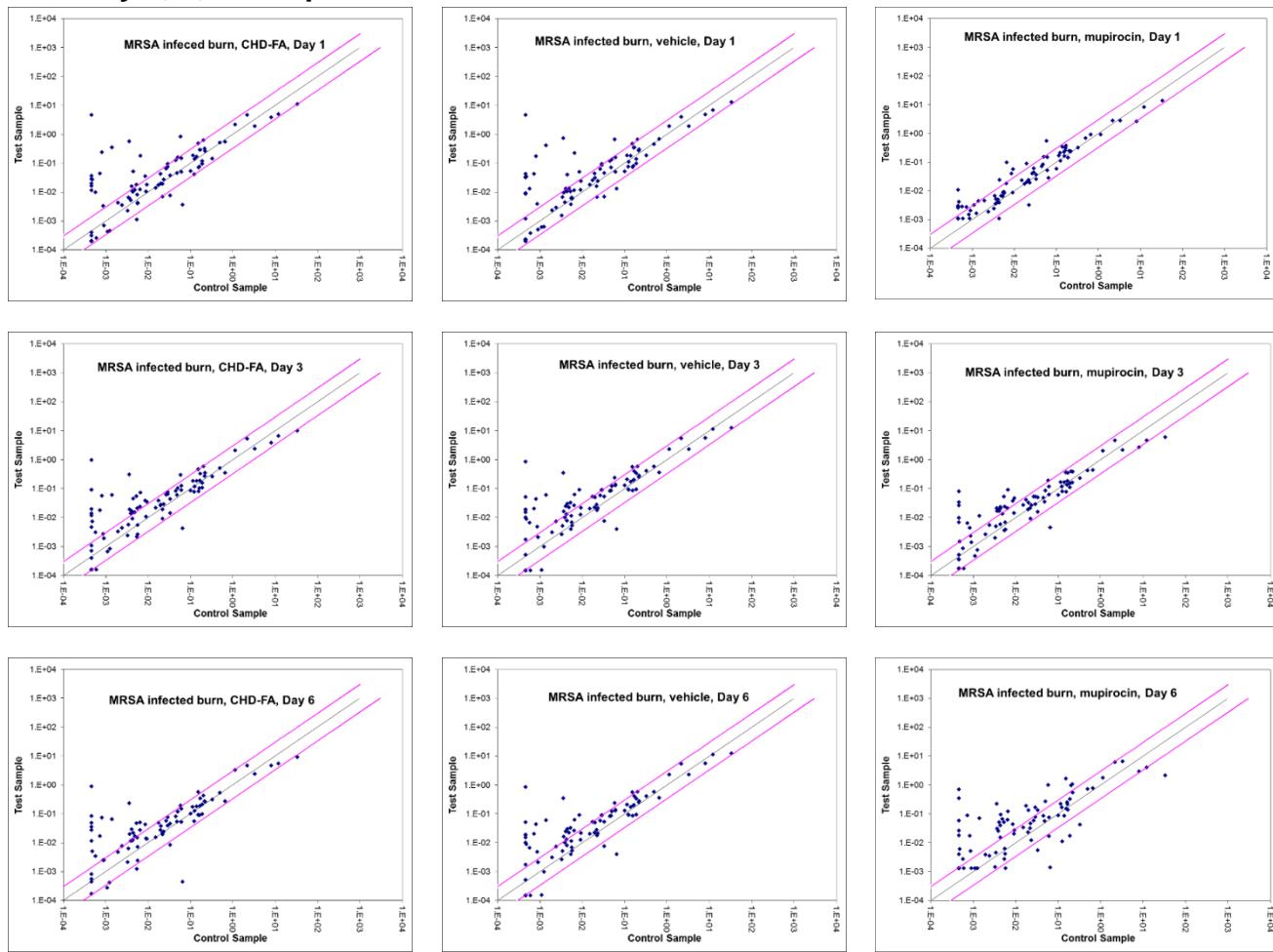
**Table 3. Histopathological evaluation scoring system (modified for burn wounds)**

Score	Neutrophils	Macrophages	Fibroblasts	Neovascularization	Epithelialization
0	No Neutrophils	No macrophages	No fibroblasts, no new collagen	None	No epithelialization
1	Scattered Neutrophils	Scattered macrophages	Increased fibroblasts, immature granulation tissue, little collagen	Rare immature vessels (<5/HPF)	Early epithelialization at edges of wound
2	Multifocal aggregates of Neutrophils	Clusters of macrophages	Increased fibroblasts, mild-moderate granulation tissue maturity, increased collagen	Moderate numbers of immature vessels (5-10/HPF)	Complete epithelialization, but not all layers
3	Diffuse infiltrates of neutrophils	Diffuse infiltrates of macrophages	Reduction in fibroblasts, Complete collagenization, mature granulation tissue	Many immature vessels >10/HPF)	Complete epithelialization

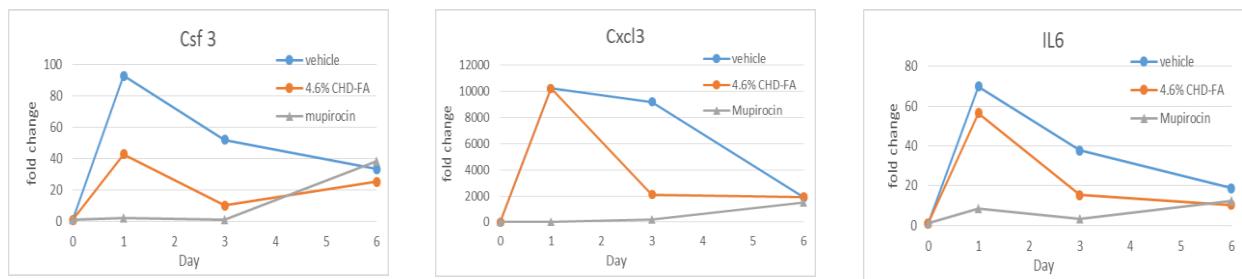
**Figure 8. Histopathological parameter kinetics among different treatment groups**



**Figure 9a. Scatter plot expression profiling of host wound healing genes along the study period at day 1, 3, and 6 post inoculation.**



**Figure 9b. Kinetic changes of representative wound healing genes**



**Table 4. Kinetic changes of prominently up-regulated wound healing genes among different treatment groups**

Genes	Mupirocin				Vehicle				4.6% CHD-FA			
	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6	Day 0	Day 1	Day 3	Day 6
Ccl12	1	23.36	57.12	57.52	1	71.90	115.04	40.79	1	78.79	42.81	106.60
Ccl7	1	14.89	13.99	18.84	1	34.73	17.34	9.58	1	27.86	11.31	7.81
Csf3	1	2.39	1.14	38.48	1	92.93	52.20	33.36	1	42.81	10.06	25.39
Cxcl1	1	3.24	8.32	51.84	1	297.76	204.51	43.11	1	250.73	43.11	46.72
Cxcl11	1	5.96	73.31	8.61	1	20.08	73.31	22.16	1	24.93	29.86	59.96
Cxcl3	1	6.62	175.58	1526.49	1	10212.16	9190.96	1884.54	1	10226.32	2105.58	1932.16
Cxcl5	1	5.64	14.89	128.53	1	70.42	55.18	41.36	1	62.25	25.46	77.49
Il10	1	4.98	1.54	4.88	1	23.07	20.62	11.88	1	17.63	5.54	6.05
Il1b	1	0.98	5.96	62.08	1	207.65	376.37	98.36	1	160.90	85.04	66.07
Il6	1	8.61	3.11	12.52	1	69.94	37.69	18.64	1	56.49	15.24	10.38
Mmp9	1	2.39	21.05	752.74	1	18.74	267.98	110.66	1	35.75	198.09	183.04
Plaur	1	3.70	8.73	119.93	1	58.81	86.58	28.25	1	60.97	24.59	22.88
Ptgs2	1	1.84	5.34	21.05	1	213.49	207.36	53.82	1	298.17	68.59	89.64
Serpine1	1	5.37	4.65	21.05	1	9.11	11.20	6.63	1	11.55	10.20	4.88
Timp1	1	9.49	3.24	17.34	1	11.54	7.65	4.06	1	14.22	5.10	3.26

#### Section 10. Evaluation of CHD-FA to treat burn wounds infected with *K. pneumoniae* strain BK#26594 blaKPC in 6 day study.

To assess the treatment efficacy of CHD-FA against cutaneous wounds infected with *K. pneumoniae* strain BK#26594 (bla<sub>KPC</sub>), 21 rats were randomized into three cohorts: 9 in CHD-FA treated, 6 in vehicle (batch #2367) controls, and 6 in antibiotic (Colistin 20 ug/ml) treatment controls (batch #SLBG4834V) at day 1, day 3, and day 6 post-inoculation time points. Carbapenem resistant *K. pneumoniae* strain BK26594 was inoculated in Brain Heart Infusion (BHI) media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 2x10<sup>5</sup> colony forming units (CFU) per ml for the infection. Two burn wounds were created on each rat, for a total of 42 wounds. The process of anesthetizing the rats and creating the burn wounds was the same as previously described in quarterly report Q9.01.2015. After burn wound creation, rats from each treatment group were infected with 0.05 ml of the *K. pneumoniae* cell suspension to a final infection dose of 1 x10<sup>4</sup> CFU. Rats were given daily treatments of antibiotic, vehicle or CHD-FA at 4.6% in 0.05 ml volumes starting at 30 min post infection. Highly purulent discharge in burn wounds was visually observed early in the infection (days 1 to 3) with the highest CHD-FA dose of 4.6%. The 4.6% CHD-FA had >10% improvement in wound closure relative to the untreated controls on days 3-6. The antibiotic control group (Colistin 20 µg/ml) had improved wound closure early in the infection with >15% closure relative to the untreated wounds on day 2.

Scab formation did not occur until day 5 in the CHD-FA and vehicle treated groups (**Fig 9**). Purulent discharge was evident in all rats throughout the study, however the duration and amount of discharge were less in the CHD-FA treated rats at 4.6% relative to the untreated controls. Bacterial burden analysis of the wounds at the experimental endpoint revealed a modest 1.6 log reduction of CFU

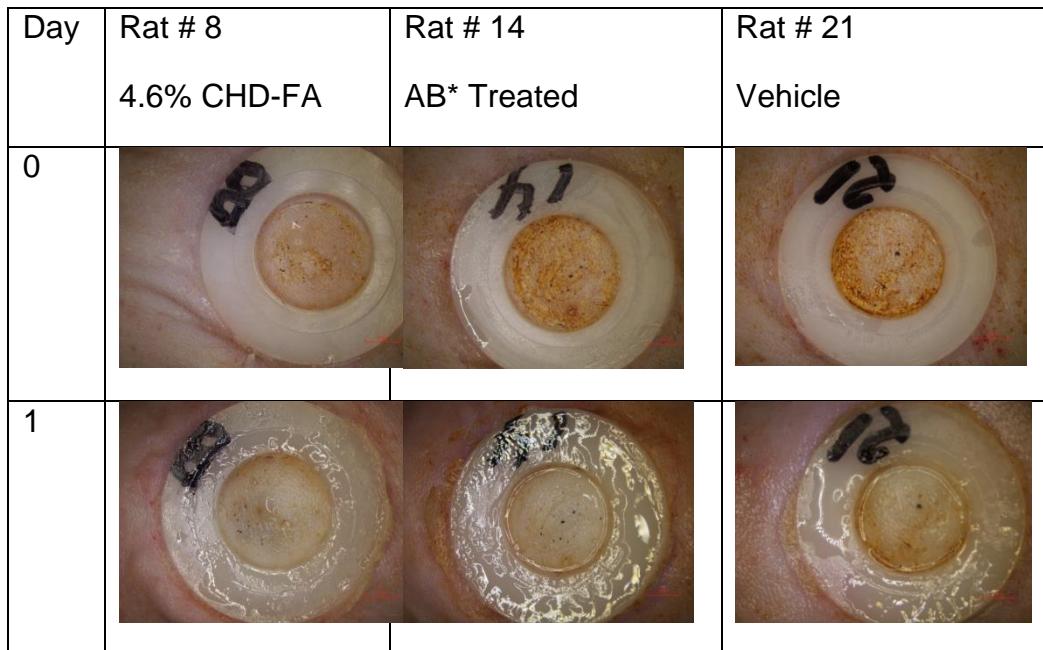
relative to the untreated control group. The other treatment doses did not significantly reduce the bacterial burden.

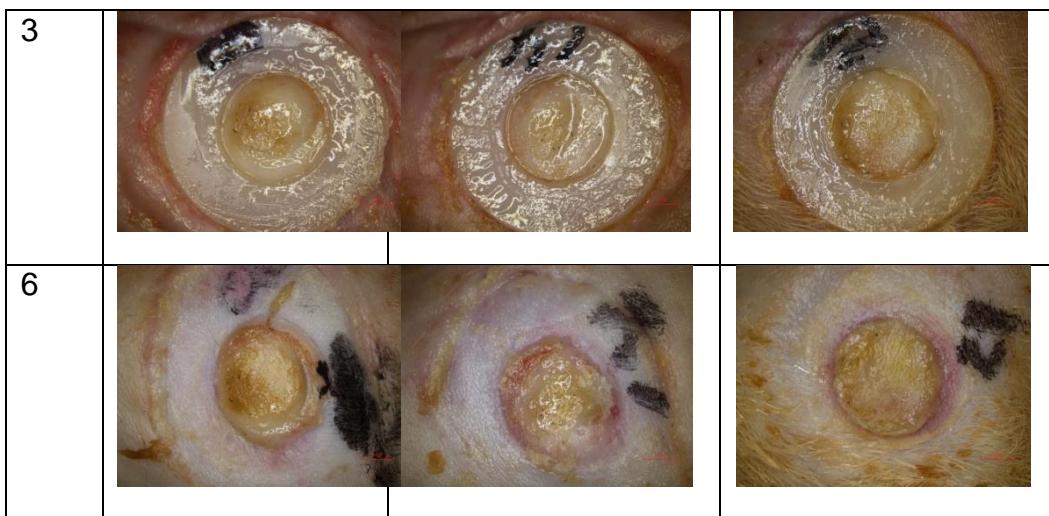
Histopathological evaluation of wounds from each treatment group was also performed throughout the study period at days 1, 3, and 6 post-inoculation. Due to the different nature of cutaneous and burn wounds, the scoring criteria was modified (**Table 6**) to best fit the burn wound healing analysis. According to this new criteria, neutrophils scores in CHD-FA treated wounds were higher at day 1 and day 3 but much lower at day 6, compared to the vehicle control, indicating a faster and better balanced inflammatory response pattern. No significant difference on scores for the presence of macrophages, fibroblasts, neovascularization, and epithelialization was observed between CHD-FA treated and untreated burn wounds in this trial.

## Conclusion

Infected wounds that were treated with 4.6% CHD-FA demonstrated improved healing over the untreated wounds by scab formation earlier and less purulent discharge. The antibiotic (20 ug/ml Colistin) treated wounds, did display a 2 log reduction of microbial burdens by day 3, however purulent discharge was also observed. The bacterial burden in the wound sites peaked at day 3 post inoculation and thereafter started to fall (See **Table 5**.) Histopathological examination on *K. pneumoniae* infected burns revealed a faster and better balanced inflammatory response and wound healing process upon CHD-FA application, consistent with previous observations in cutaneous wounds and burns.

**Figure 9. Burn wound images of rats infected with *K. pneumoniae* (BK#26594) and treated with CHD-FA at 30min post inoculation.**





\*20.0 ug/ml Colistin

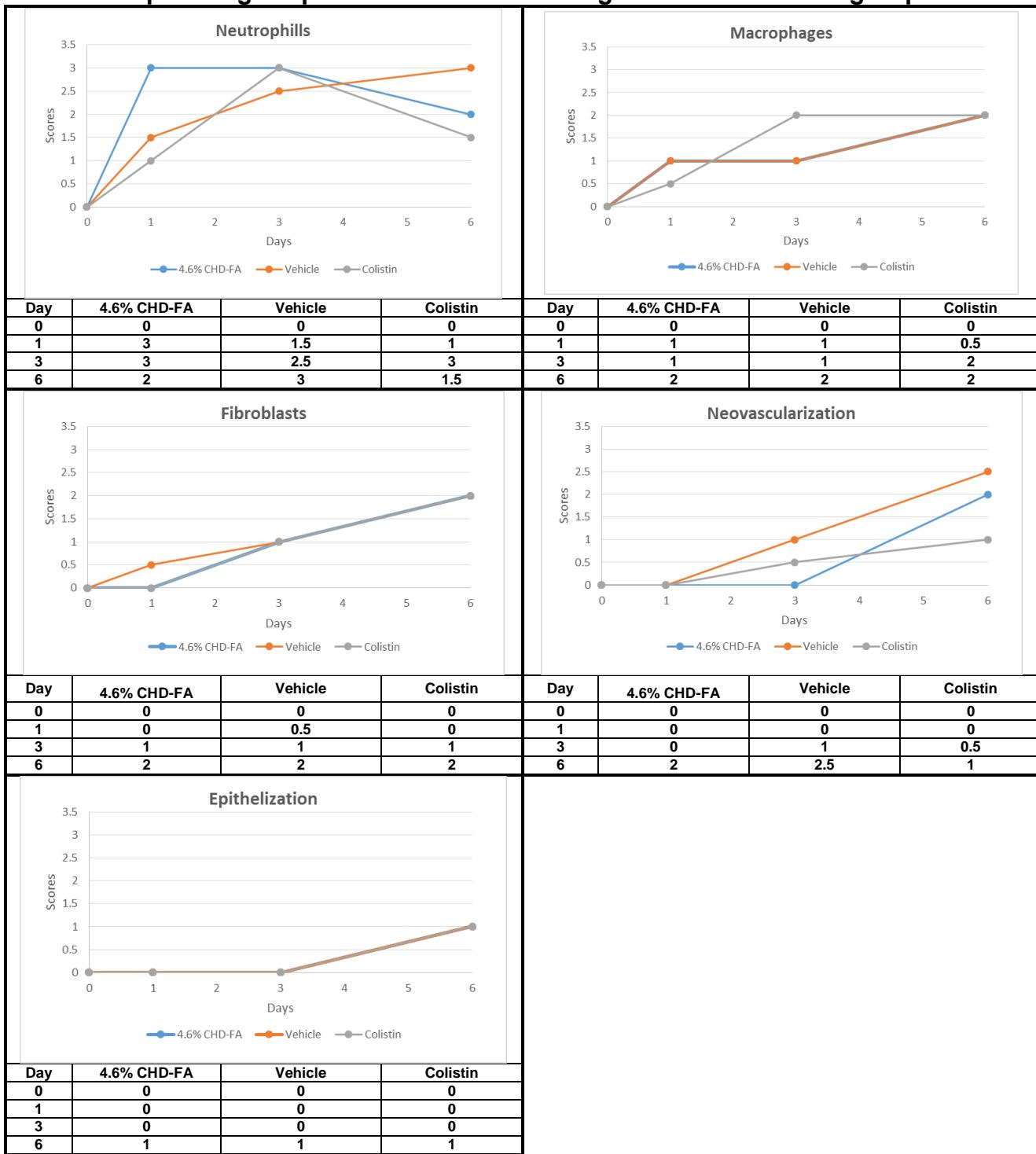
**Table 5. Bacterial burdens of burn wounds infected with *K. pneumoniae* at Day 1, Day 3, and Day 6 experimental endpoints.**

<i>K. pneum</i> $10^4$ CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	7.7	7.5-7.8	NA	8.8	8.5-9.1	NA	8.5	NA	NA	<i>K. pneum</i> $10^4$ CFU 0.5h Tx
4.6%	8.3	8.1-8.5	-0.6	8.5	8.4-8.5	0.3	8.1	7.6-8.6	0.4	
AB Control	5.7	5.3-6.1	2.0	6.8	5-8.5	2.0	NA	NA	NA	

**Table 6. Histopathological evaluation scoring system (modified for burn wounds)**

Score	Neutrophils	Macrophages	Fibroblasts	Neovascularization	Epithelialization
0	No Neutrophils	No macrophages	No fibroblasts, no new collagen	None	No epithelialization
1	Scattered Neutrophils	Scattered macrophages	Increased fibroblasts, immature granulation tissue, little collagen	Rare immature vessels (<5/HPF)	Early epithelialization at edges of wound
2	Multifocal aggregates of Neutrophils	Clusters of macrophages	Increased fibroblasts, mild-moderate granulation tissue maturity, increased collagen	Moderate numbers of immature vessels (5-10/HPF)	Complete epithelialization, but not all layers
3	Diffuse infiltrates of neutrophils	Diffuse infiltrates of macrophages	Reduction in fibroblasts, Complete collagenization, mature granulation tissue	Many immature vessels >10/HPF)	Complete epithelialization

**Figure 10. Histopathological parameter kinetics among different treatment groups**



## Section 11. Efficacy of a pH stabilizing buffered CHD-FA gel to treat open cutaneous wounds infected with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain Xen31.

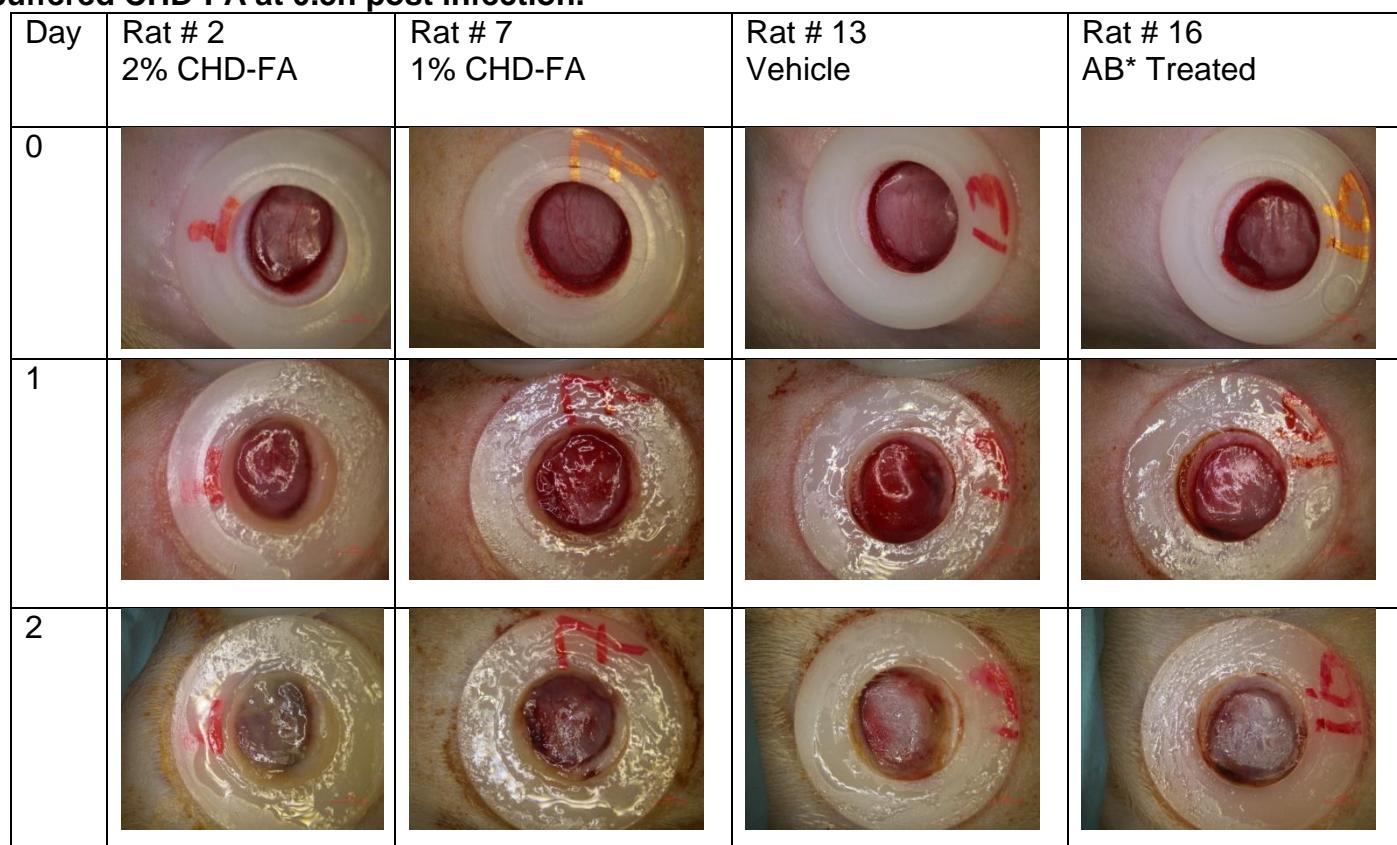
To better assess the treatment efficacy of CHD-FA in early stage of infection against cutaneous wounds infected with MRSA, the wound study was repeated with the MRSA Xen31 strain as previously described in the Q3 report. Wounds were created and infected with 0.05 ml of MRSA to a final infection dose of 100 CFU per wound. Cohorts of 18 rats were randomized into 2% buffered CHD-FA gel (n=6), 1% buffered CHD-FA gel (n=6), vehicle control (n=3) and antibiotic control (2%

Mupirocin cream) (n=3). Rats were given twice daily treatments starting at 30min followed by 8h post inoculation. Twice daily treatments were included in this study in order to evaluate the progress of wound healing early in infection. Microbial burdens were assessed on the wounds from the CHD-FA, antibiotic and sham treated groups at 4hr, day1 and day 2 time point. Purulent discharge was observed in all of the CHD-FA treated and untreated rats, although the discharge was less pronounced in the CHD-FA treated groups. However, No burden reduction was observed from either of pH buffered CHD-FA gel formulation (**Figure 11, 12, and Table 7**). Rapid growth of bacteria multiplying by  $>10^4$  fold within 24h was observed for both CHD-FA groups, although the burden development slowed down from 24h to 48h post-inoculation. In contrast, inoculated bacteria were completely eradicated from wounds in rats treated with mupirocin cream as early as 4h post-inoculation and kept clean throughout the entire experiment period.

## Conclusion

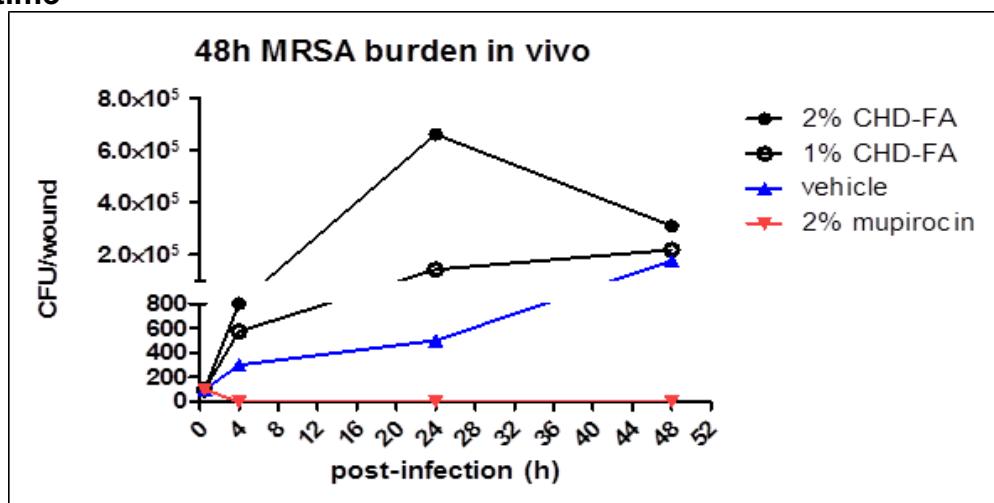
No significant changes in microbial burden reduction were observed in this trial as observed previously in the cutaneous wound infection model. The pH stability of the CHD-FA with the gel formulation may still be insufficient in the wound.

**Figure 11. Wound images of rats infected with  $10^2$  CFU/mL MRSA (Xen31) and treated with buffered CHD-FA at 0.5h post infection.**



AB : 2% mupirocin cream

**Figure 12. Bacterial burdens of cutaneous wounds infected with MRSA vs. post-inoculation time**



**Table 7. Bacterial burdens of cutaneous wounds infected with MRSA at 4h, Day 1, and Day 2 experimental endpoints.**

MRSA 10 <sup>2</sup> CFU	4 Hour			Day 1			Day 4			Study
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	MRSA 10 <sup>2</sup> CFU 0.5h Tx
No Tx Control	2.7	NA	NA	3.0	NA	NA	5.5	NA	NA	
2%	2.9	NA	-0.2	5.5	NA	-2.5	5.5	5.1-5.9	0.0	
1%	2.8	2.7-2.8	-0.1	4.8	4.7-5.0	-1.8	5.4	NA	0.1	
AB Control	NA	NA	NA	NA	NA	NA	NA	NA	NA	

### Section 12. Evaluation of a topical cream-based CHD-FA to treat cutaneous open wounds infected with low dose MRSA in 48 hr study.

The purpose of this trial was to test if a topical cream-based CHD-FA formulation has better absorptive properties and pH stabilizing properties compared to the gel formulation. Three rats were randomized in three cohorts: vehicle (batch#2367) treated, 2% mupirocin cream treated, and CHD-FA cream (batch#P124/14) treated at 30min post-inoculation time point on a daily basis for 48h.

Methicillin Resistant *S. aureus* strain MW2 was inoculated in BHI media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to infect the wounds at  $2 \times 10^5$  CFU per ml for the infection. Two open wounds were created on each rat, for a total of 6 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of  $1 \times 10^4$  CFU. Rats were given corresponding treatments in 50  $\mu$ l volumes starting at 30min post infection. The 30min treatment starting time was used to determine if significant improvement in burden reduction would be observed.

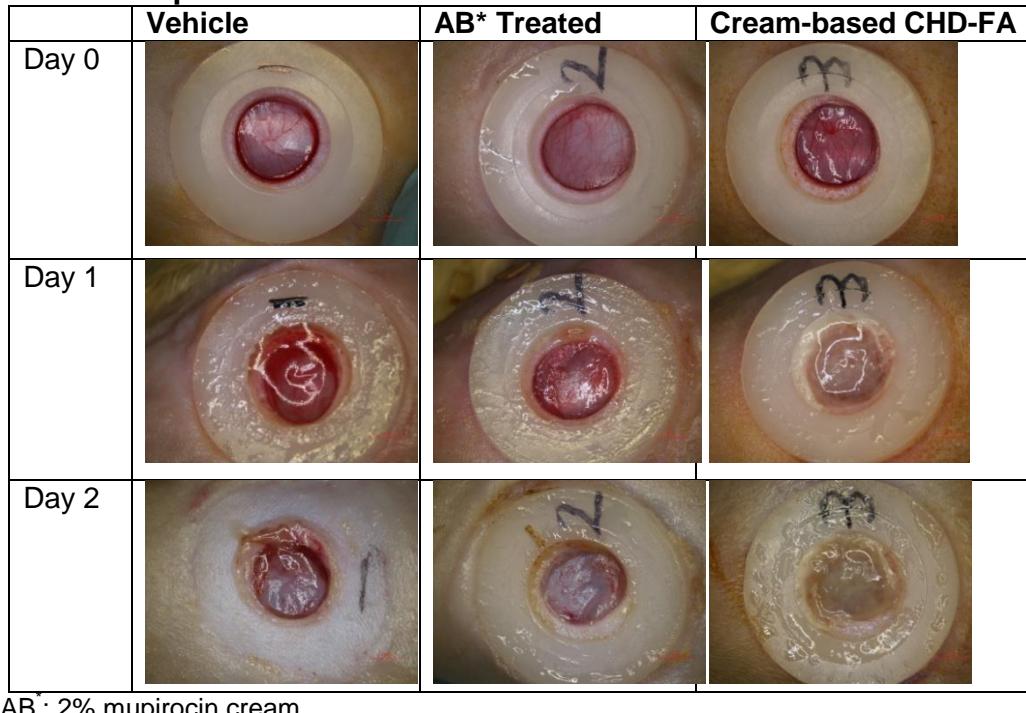
The bacterial burden in the CHD-FA cream treated wound sites was not reduced relative to vehicle control, which was possibly due to the heavy biofilm formed on the wound bed preventing absorption of active compound (See **Figure 13.**) Also the topical cream formulation has been approved for

vaginitis to help control inflammation related symptoms which may not provide pH stability needed for cutaneous wounds.

## Conclusion

The topical cream formulated CHD-FA treatment on low dose MRSA infected wounds did not improve the wound healing or show significant reduction in microbial burden. The pH stability of the CHD-FA with the in topical cream formulation may still be insufficient in the wound.

**Figure 13. Wound images of MRSA infected cutaneous open wound and treated with cream based CHD-FA at 30min post infection.**



## Section 13. Evaluation of pH changes in CHD-FA treated cutaneous wounds.

The pH effects on the bactericidal properties of CHD-FA *in vitro* suggested that it may be a possible cause of the microbial burden persistence in previous cutaneous wound infection studies as previously described in quarterly report Q9.01.2015.

Therefore, the dermal pH value in the open wound of rat skin was measured *in vivo* using a pH micro-electrode over a course of 24h. The purpose of this pilot trial was to explore the impact of CHD-FA treatment on the pH of the wound bed and its implications on bactericidal properties of CHD-FA.

The pH meter EL20/EL2 (Mettler-Toledo, Schwerzenbach, Switzerland) combined with pH micro-electrode (Warner Instruments, Hamden, CT) were used to measure the pH of the extracellular dermis. The pH micro-electrode was calibrated in two standard pH buffer solutions (Hanna Instruments, Woonsocket, RI) before each measurement. Standard buffer A had a pH of  $4.01 \pm 0.01$  at  $25^{\circ}\text{C}$  and standard buffer B had a pH of  $7.01 \pm 0.01$  at  $25^{\circ}\text{C}$ .

Three rats were randomized into three groups: 1 rat infected and treated, 1 rat infected but untreated, and 1 rat uninfected with right wound treated and left wound untreated.

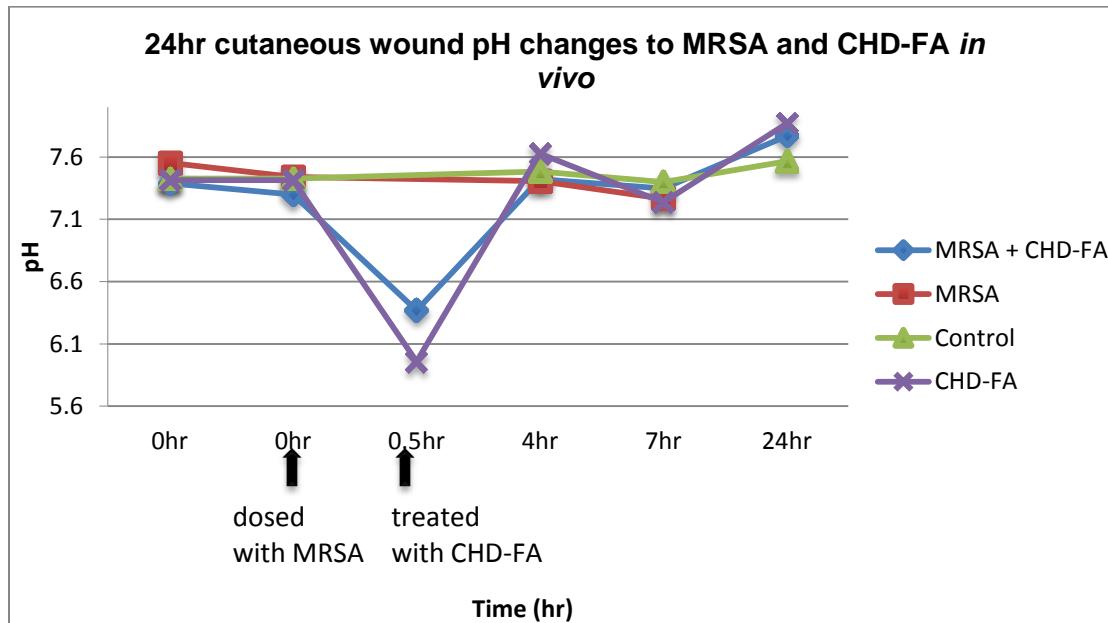
MRSA strain MW2 was prepared as described above in section 2d on evaluating CHD-FA cream. Two cutaneous wounds were created on each rat, for a total of 6 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from the infection group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of  $1 \times 10^4$  CFU. Rats from the treatment group were given 4.6% CHD-FA gel in 50  $\mu$ l volumes starting at 30min post infection.

Wound pH measurement was performed prior and at 0.5, 4, 7, and 24h post CHD-FA treatment. The pH was at  $7.44 \pm 0.07$  on uninfected wounds, and was a little lower at  $7.37 \pm 0.10$  once infected. Upon CHD-FA treatment, pH dropped to 6.37 on infected wound and 5.96 on uninfected wound shortly at 0.5 h post treatment, but it quickly restored basic to an average of  $7.50 \pm 0.10$  for all types of wounds at 4h post treatment and stabilized thereafter (**Table 8, Figure 14**). This observation suggests that formulation modification may be required to maintain the low pH in wound sites to improve *in vivo* antimicrobial property of CHD-FA.

**Table 8. The pH of cutaneous wound bed *in vivo* with MRSA and CHD-FA at 0h, 0.5h, 4h, 7h, and 24h**

	0hr	0hr (after MRSA infection)	0.5 hr (after CHD-FA treatment)	4hr	7hr	24hr
<b>MRSA + CHD-FA</b>	7.39	7.30	6.37	7.42	7.35	7.77
<b>MRSA</b>	7.55	7.44		7.41	7.27	
<b>Control</b>	7.43			7.48	7.40	7.57
<b>CHD-FA</b>	7.41		5.96	7.63	7.24	7.87

**Figure 14. The pH of cutaneous wound bed *in vivo***



## **Section 14. Serum impact on *in vitro* efficacy of CHD-FA**

To evaluate serum impact on *in vitro* antimicrobial potency of CHD-FA in the pH stabilizing gel formulation, we have performed MIC testing for MRSA strain ATCC33591 in the presence of rat serum and human serum (Sigma Human Serum - catalog # H4522-100ml 025K0462 – Human serum from human male AB plasma.)

CHD-FA was supplied as a 4.6 % solution (Jan 2013 batch) and 4.6% Gel (Aug 2014 batch) by Fulvimed (Pty) Ltd (A division of Fulhold Ltd.), Somerset West, South Africa. CHD-FA was stored in the dark at room temperature. A highly standardized broth-based *in vitro* susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” was used to determine the MIC values in the presence of 50% serum. Briefly, bacterial colonies from a freshly grown agar plate were re-suspended in CLSI recommended cation-adjusted Mueller Hinton broth for aerobic bacteria. MIC values were visually read at 48h. The MIC is defined as the lowest concentration producing a prominent decrease in turbidity compared to the drug free well. All assays were performed in duplicate.

The 4.6% CHD-FA gel MIC in the presence of rat serum is 0.06%, human serum 0.12% and no serum 0.06% at 48h. Similarly, the 4.6% CHD-FA solution MIC in the presence of rat serum is 0.13%, human serum 0.06% and no serum 0.06% at 24h.

Overall, there was no significant change in the MIC against MRSA treated with CHD-FA in the presence of rat or human serum. (See **Table 9.**)

**Table 9. MIC of MRSA ATCC33591 to CHD-FA in the presence of rat serum, human serum**

MRSA-ATCC33591	Type of Serum	MIC at 48h
4.6% CHD-FA gel- pH 4.5	Rat	0.12%
	Human	0.25%
	None	0.12%
4.6% CHD-FA solution- pH 1.7	Rat	0.25%
	Human	0.13%
	None	0.06%

## **Section 16. Evaluation of CHD-FA promoting wound healing of burns in the absence of infection**

To understand the function of CHD-FA on burn wound healing in the absence of infection, we compared the healing process of burns treated with CHD-FA and vehicle by wound image, histopathology evaluation, and expression profiling of wound healing genes. A total of 9 rats were randomized into four groups: 6 in 4.6% CHD-FA group, and 3 in untreated group. The process of anesthetizing the rats and creating the burn wounds was the same as previously described in

quarterly report Q10.04.2015. Rats from the treatment group were given daily treatments of CHD-FA at 4.6% in 0.05 ml volumes starting at 30 min after the wound creation. **Figure 16** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 6. Burns treated with CHD-FA did not look much different from untreated.

Histopathological evaluation was performed on samples collected throughout the study period from day 1 to day 6. While consistency of burn creation was confirmed using this method, healing assessment parameters were not significantly different between CHD-FA treated and untreated burns (**Figure 17**).

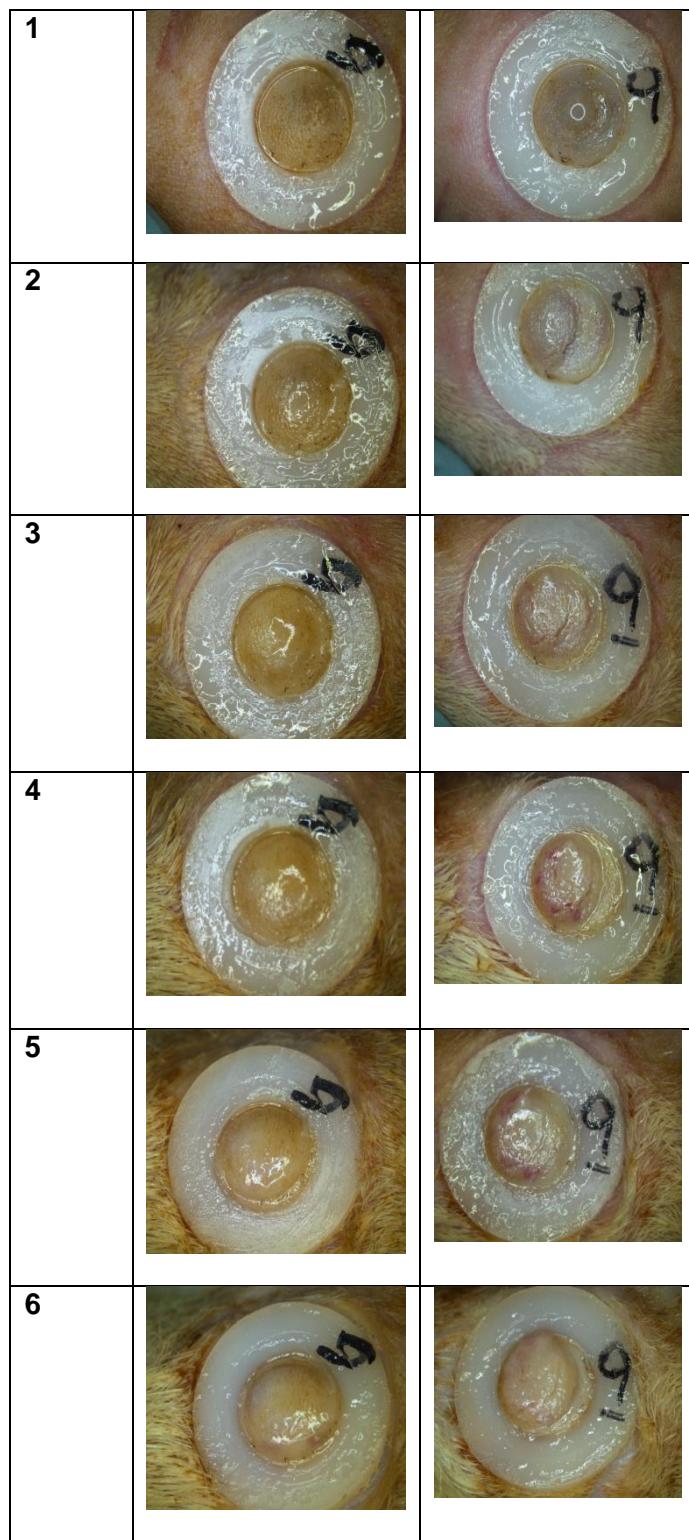
Wound healing gene expression profiling was performed using a commercial PCR array (The Rat Wound Healing RT<sup>2</sup> Profiler™ PCR Array, Cat No. 330231, Qiagen). In the absence of infection, prominent expression change was observed with almost the same panel of genes as previously described for burns infected with MRSA (Q10 04.15.15), but in general the differential of gene expression change was lower than the infected case. As **Figure 18a** illustrated, expression profiles were very similar between CHD-FA treated and untreated burns at all three time points. However, it is noticeable that two most commonly used biomarkers to assess burn wound healing, MMP-9 and TNF (tumor necrosis factor), were found to be controlled at a significantly lower level in CHD-FA treated burns compared to untreated (**Figure 18b**). MMP-9 is matrix metalloproteinase to function in tissue remodeling and TNF is a pro-inflammatory factor. Overexpression of MMP-9 and TNF have been related to delayed burn wound healing, vice versa, treatments that reduce expression of these biomarkers demonstrated accelerated wound healing [1, 2]. Hence, the noted expression profile of MMP-9 and TNF in CHD-FA treated burn wounds displayed a faster transition from inflammation to tissue remodeling induced by CHD-FA. The other important wound healing biomarker IL-6 was not remarkably different between treated and untreated wounds (**Figure 18b**).

## Conclusion

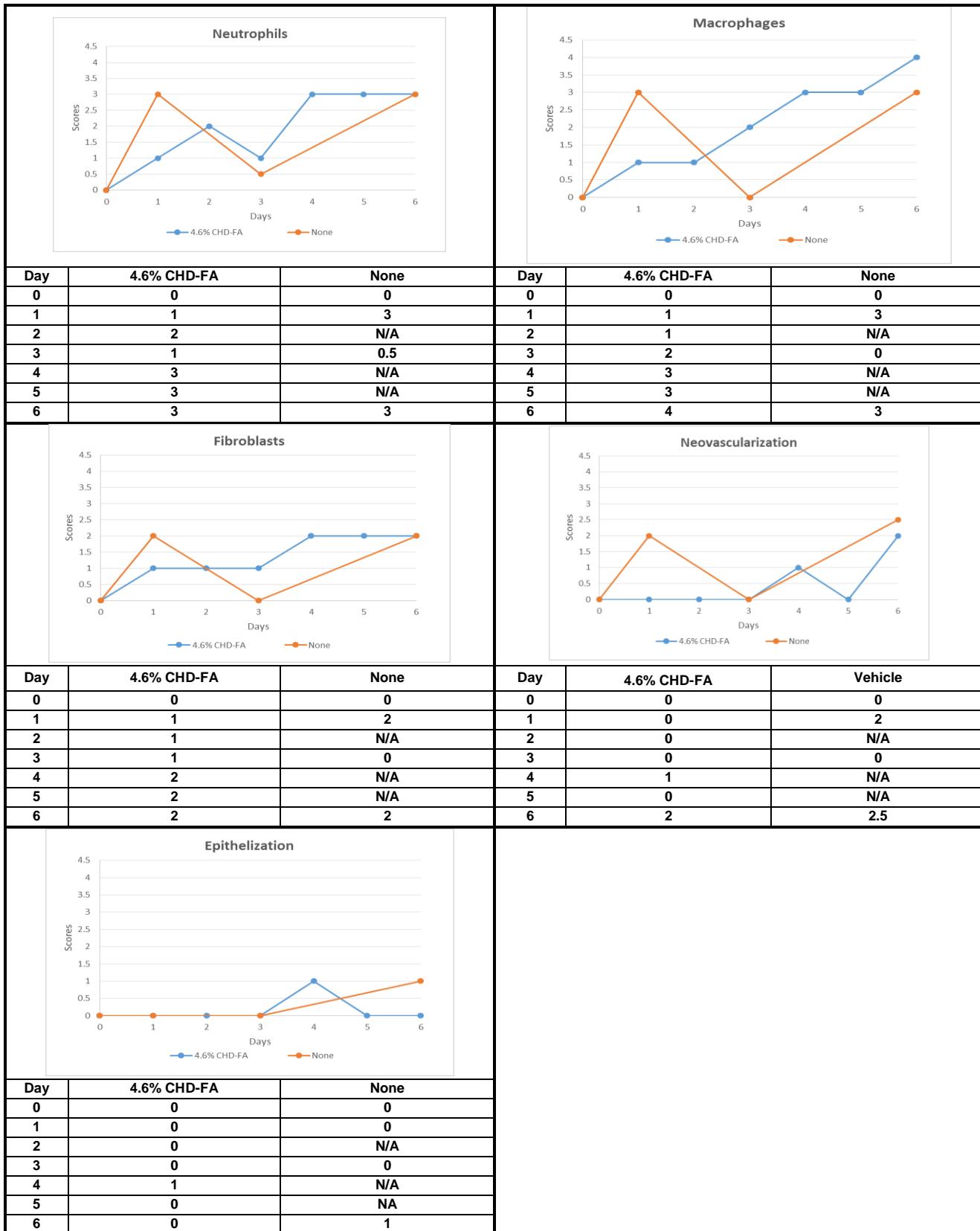
Although the healing process did not look much different by wound image and histopathological evaluation. Expression profiling did support that CHD-FA is promoting burn wound healing in the absence of infection. The anti-inflammatory effect of CHD-FA is robust and highly reproducible.

**Figure 16. Burn wound images of burn wounds untreated and treated with CHD-FA**

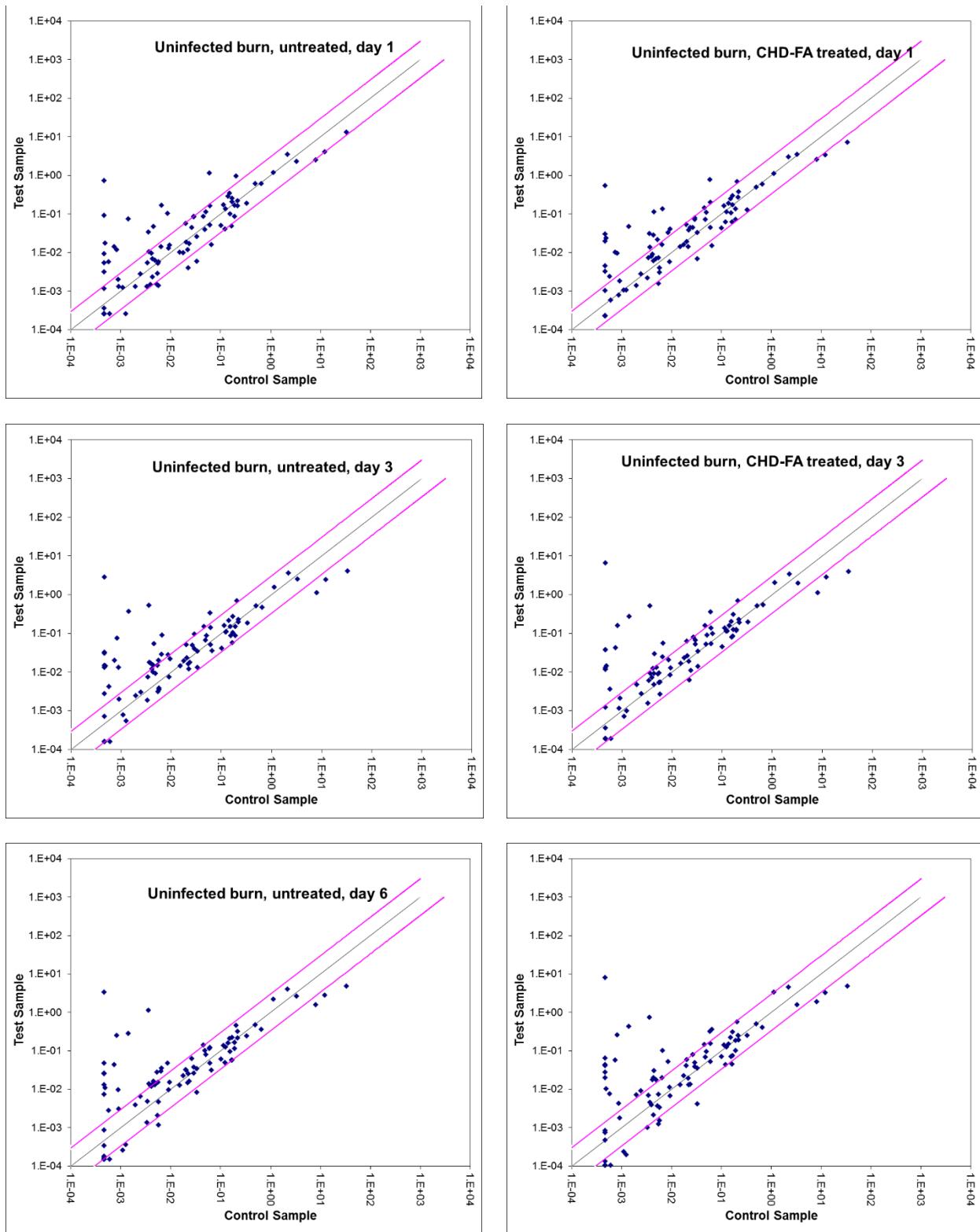
Day	CHD-FA	None
0		



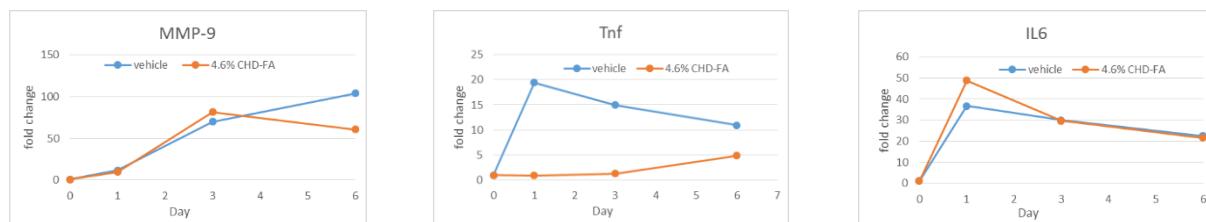
**Figure 17. Histopathological parameter kinetics among different treatment groups**



**Figure 18a. Scatter plot expression profiling of host wound healing genes along the study period at day 1, 3, and 6.**



**Figure 18b. Kinetic changes of MMP-9 and TNF**



## Section 17. Improved burn wound creation confirmed by histopathological evaluation

To establish an accurate model for the burn wound infection, we created a reproducible model for creating a full-thickness 3<sup>rd</sup> degree burns. The depth and intensity of burn can impact bacteria's ability to thrive on burnt skin surface, and heterogeneity of burn creation can result in a biased healing assessment. We have noticed some heterogeneous burn creation by histopathological examination in previous experiments. Therefore, we have modified our burn creation method by applying a constant pressure on the soldering handle. A total of 4 burn wounds were created in 2 rats for this purpose.

**Figure 19** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 2. 2 burn wounds were harvested at 24h and the other 2 wounds at 48h for histopathological evaluation of the consistency in depth of the burn (**Figure 20**).

## Conclusion

According to the histopathological analyses, our modified method of creating a burn by using a constant pressure resulted in a better uniformity of the depth. This modified method will be used for future burn model infection experiments to test the efficacy of CHD-FA in different challenge pathogens.

**Figure 19. Uninfected burn wound images of rats**

	Uninfected Rat#1 Left	Uninfected Rat#1 Right	Uninfected Rat#2 Left	Uninfected Rat#2 Right
<b>Day 0</b>				

Day 1			NOT PICTURED	NOT PICTURED
Day 2	EUTHANIZED ON DAY1	EUTHANIZED ON DAY1		

**Figure 20. Histopathological analysis of burn wounds**

Rat#1 Left	Rat#1 Right	Rat#2 Left	Rat#2 Right
Cell and tissue death extends down to the dermis/hypodermis junction (superficial aspects of hair bulbs necrotic). Epidermis is intact with discernable nuclei and rare small foci of bacteria. Superficial 2/3 of dermis denatured with minimal inflammation. There is hypodermal edema	Cell and tissue death extends down through the muscle layer. The epidermis is intact and contains a few vesicles with proteinaceous material and rare foci of bacteria. Dermis denatured with minimal inflammation. There is hypodermal edema and degeneration of muscle cells/fibers. There is mild diffuse subdermal edema with increased numbers of fibroblasts and lymphocytes and vessels with plump endothelial cells.	Cell and tissue death extends down midway through the muscle layer. The epidermis is intact with scattered accumulations of bacterial colonies between and on the cell layers as well as in hair follicles. Dermis denatured with minimal inflammation. There is hypodermal edema and degeneration of muscle cells/fibers with an influx of lymphocytes and fewer numbers of macrophages and fibrocytes at the margin between viable and necrotic tissue. There is moderate diffuse subdermal edema with markedly increased numbers of fibroblasts and vessels with plump endothelial cells.	Cell and tissue death extends down through dermis and small amount of muscle. Epidermis is intact and epidermal cell nuclei still discernable with scattered accumulations of bacterial colonies between and on the epidermal cell layers as well as in hair follicles. The superficial dermis is multifocally disrupted with separation of collagen fibers. There is hypodermal and muscle layer edema and inflammation with most inflammation occurring in the subcuticular matrix.

#### **Section 18. 2d. Evaluation of new CHD-FA formulations to treat cutaneous wounds infected with low dose of MRSA in 48 hr study.**

To resolve the lack of *in vivo* antimicrobial activity of CHD-FA, our commercial partner provided three new formulations for burden reduction evaluation, including CHD-FA zinc diacetate gel, CHD-FA tea tree oil, and CHD-FA thymol gel. A total of 10 rats were randomized into five treatment cohorts: saline, 2% mupirocin cream, CHD-FA zinc diacetate gel (batch#2815), CHD-FA tea tree oil (batch#2816) , and CHD-FA thymol gel (batch#2814). All treatments were initiated at 30min post-inoculation and applied on a daily basis. At 24h post-inoculation, one rat from each treatment group was sacrificed and the rest of rats were sacrificed at 48h post-inoculation. Wounds were dissected, homogenized and plated for burden quantification. Daily wound image was taken for all rats (**Figure 21**). Methicillin Resistant *S. aureus* strain MW2 was inoculated in BHI media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $1 \times 10^4$

CFU/ml. Two open wounds were created on each rat, for a total of 20 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of 500 CFU. Rats were given corresponding treatments in 0.05 ml volumes starting at 30min post inoculation. The 30min treatment starting time was used to determine if significant improvement in burden reduction would be observed.

Unlike previously used gel formulation, a rapid infection site sterilization was observed with wounds treated with CHD-FA zinc formulation, whereas other formulations of CHD-FA failed to demonstrate such potency (**Table 11, and Figure 21**). The bacterial burden assessment showed 4.9, and 6.9 log burden reduction in the wounds for the CHD-FA-Zn treated group relative to the untreated controls at 24h and 48h, respectively (**Table 11**).

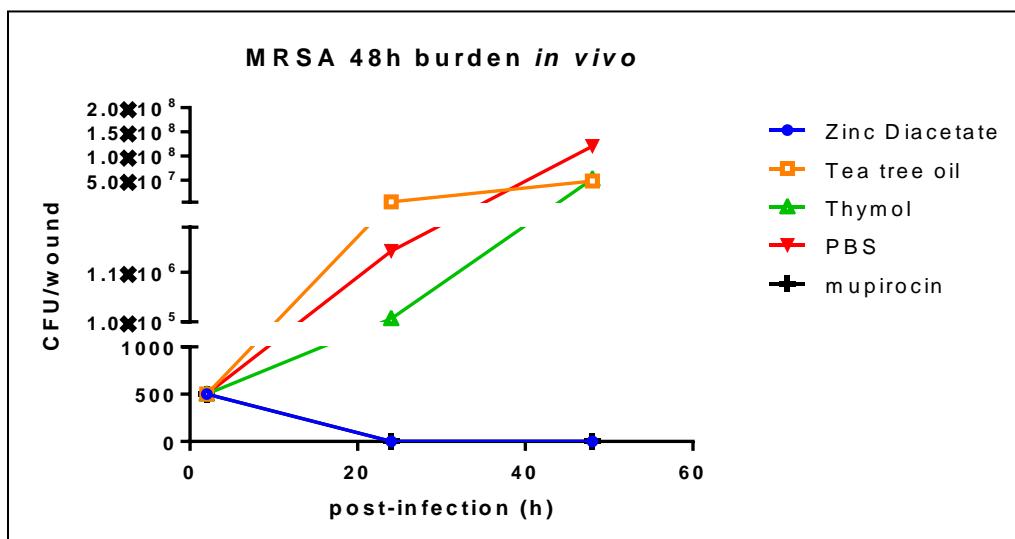
## Conclusion

Application of zinc formulated CHD-FA on low dose MRSA infected wounds resulted in a rapid wound sterilization. CHD-FA-Zn is by far the most promising formulation, and future experiments will be focused on analyzing efficacy of this specific formulation.

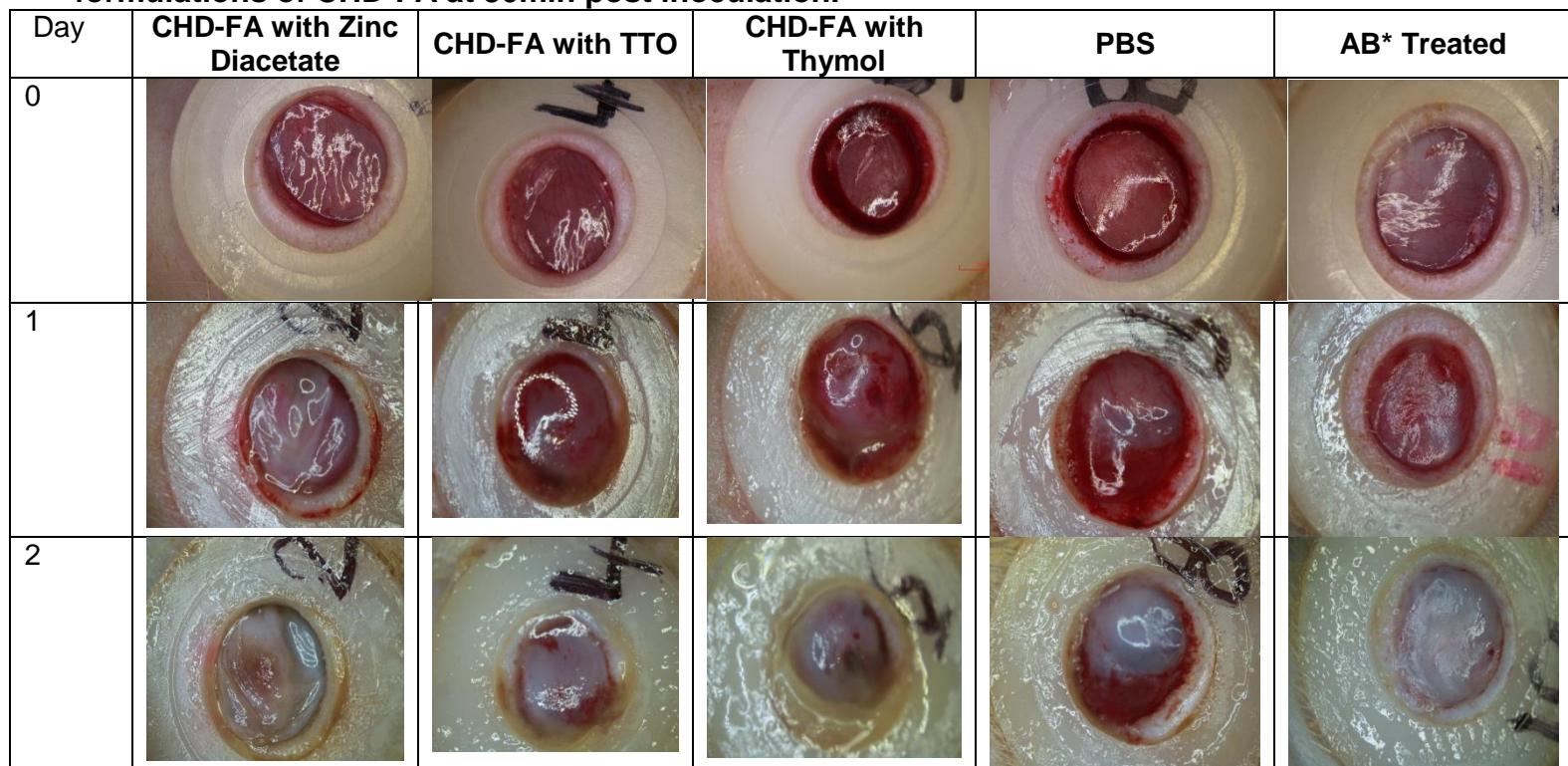
**Table 11. Bacterial burdens of burn wounds infected with MRSA at Day 1, and Day 2 experimental endpoints.**

MRSA 500 CFU	Day 1			Day 2			Study
	Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	
No Tx Control		4.9	NA	NA	6.9	NA	MRSA 500 CFU 0.5h Tx
CHD-FA with Zinc Diacetate	Sterilized		NA	NA	Sterilized	NA	
CHD-FA with TTO		5.4	NA	-0.5	6.4	NA	
CHD-FA with Thymol		3.9	NA	1.0	6.4	NA	
AB Control	Sterilized		NA	NA	Sterilized	NA	

**Figure 21. Bacterial burdens of cutaneous wounds infected with MRSA vs. post-inoculation time**



**Figure 22. Wound images of MRSA infected cutaneous open wounds and treated with different formulations of CHD-FA at 30min post inoculation.**



AB : 2% mupirocin cream

### **Section 19. Evaluation of CHD-FA-Zn to treat cutaneous wounds infected with low dose of *P. aeruginosa* in 48 hr study.**

To assess the treatment efficacy of CHD-FA-Zn against cutaneous wounds infected with *P. aeruginosa* Xen5. A total of 6 rats were randomized into two treatment groups: 2 in CHD-FA-Zn (batch#2815) group, 2 in PBS group, and 2 in colistin group. All rats were treated in 0.05ml volume at 30min post-inoculation time point on a daily basis for 48h.

*P. aeruginosa* Xen5 was inoculated in LB media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to 10<sup>4</sup> CFU per ml for the infection. Two open wounds were created on each rat, for a total of 12 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the *P. aeruginosa* cell suspension to a final infection dose of 500 CFU. Rats were given corresponding treatments in 0.05 ml volumes starting at 30min post inoculation.

Compared to PBS controls, less discharge was observed for wounds treated with CHD-FA-Zn and colistin control throughout the study period (**Figure 24**). The bacterial burden assessment found a significant burden reduction from wounds treated with CHD-FA-Zn at 24h post-inoculation, whereas untreated wound burden reached to 5.7 log CFU (**Figure 23, table 12**). However, the “sterilization” was overridden by rapidly growing *P. aeruginosa* cells at 48h post-inoculation, leaving a 4.3 log burden on the wound (**Table 12**). In comparison, colistin was still potent enough to keep the wound sterile, but bacterial burdens on untreated wounds already climbed up to >7 logs.

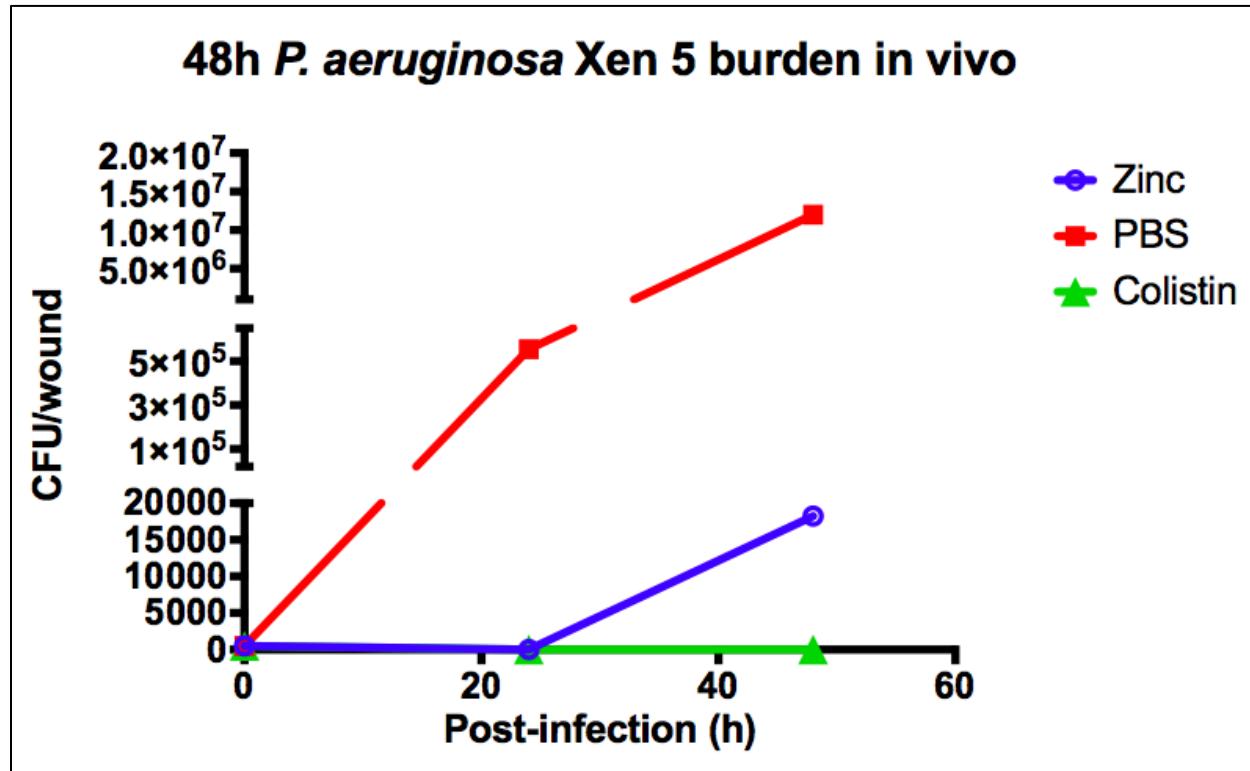
## Conclusion

To rule out individual animal variance induced error in burden assessment, we will be repeating these studies to assess the bacterial burden on the later time points. Meanwhile, we realize that single application of the CHD-FA-Zn may not be sufficient to control infections caused by virulent and rapid growing bacterial species such as *P. aeruginosa*. We will consider multiple application or change the application method (dressing) in the future experiments.

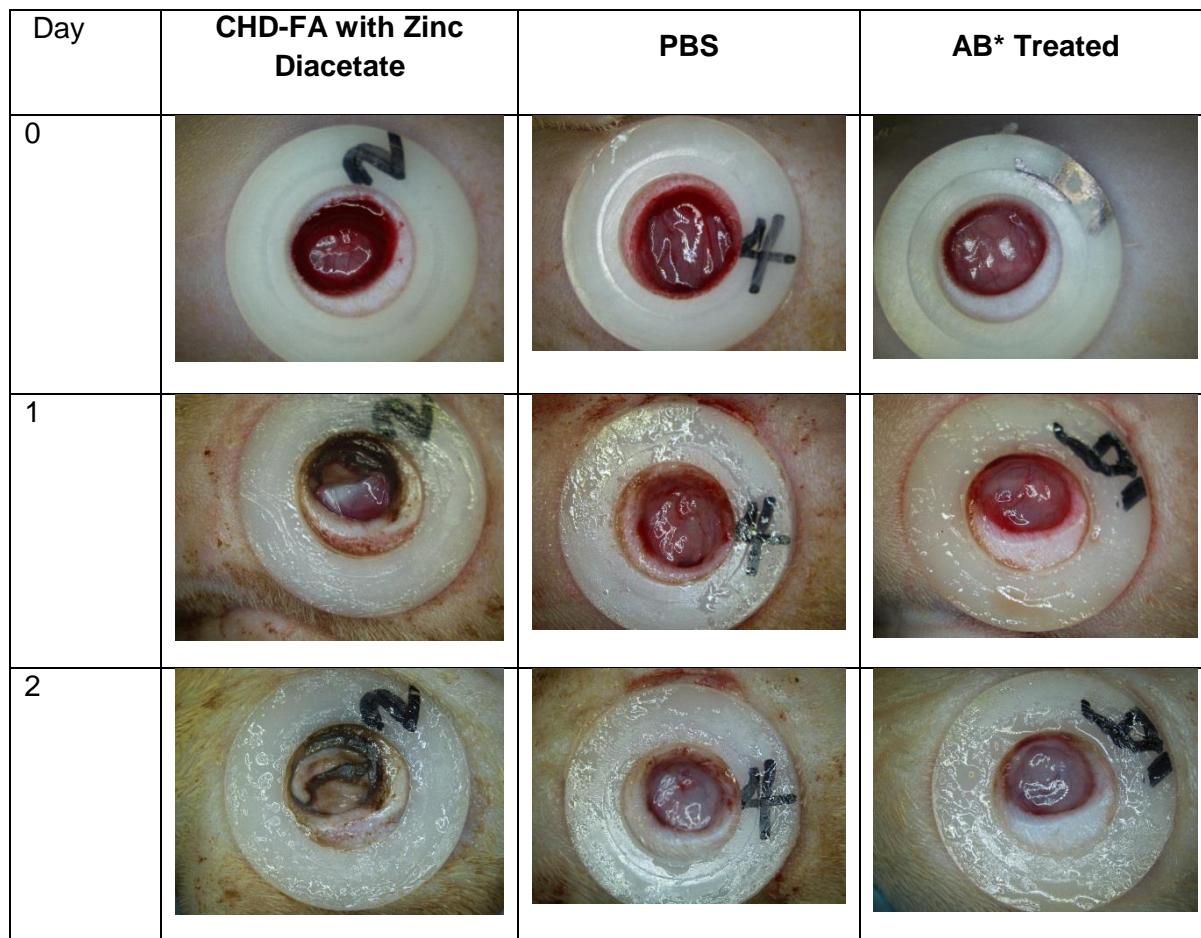
**Table 12. Bacterial burdens of burn wounds infected with *P. aeruginosa* at day 1, and day 2 experimental endpoints.**

<i>P. aeruginosa</i> 500 CFU	Day 1			Day 2			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	5.7	NA	NA	7.1	NA	NA	<i>P. aeruginosa 500 CFU 0.5h Tx</i>
CHD-FA wth Zinc Diacetate	Sterilized	NA	NA	4.3	NA	2.8	
AB Control	Sterilized	NA	NA	Sterilized	NA	NA	

**Figure 23. Bacterial burdens of cutaneous wounds infected with *P. aeruginosa* vs. post-inoculation time**



**Figure 24. Wound images of *P. aeruginosa* infected cutaneous open wounds and treated with CHD-FA zinc diacetate at 30min post infection.**



AB<sup>+</sup>: Colistin

## Section 20. Serum impact on *in vitro* efficacy of CHD-FA-Zn

To evaluate serum impact on *in vitro* antimicrobial potency of CHD-FA in the original solution and zinc formulations, we have performed MIC testing for *P. aeruginosa* Xen 5 in the presence of human serum (Sigma Human Serum - catalog # H4522-100ml 025K0462 – Human serum from human male AB plasma.)

CHD-FA was supplied as a 4.6 % solution (Jan 2013 batch) and 4.6% Gel with Zinc (June 2015 batch) by Fulvimed (Pty) Ltd (A division of Fulhold Ltd.), Somerset West, South Africa. CHD-FA was stored in the dark at room temperature. A highly standardized broth-based *in vitro* susceptibility assay following the Clinical and Laboratory Standards Institute (CLSI) protocol M07-A9 “Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard – Eighth Edition” was used to determine the MIC values in the presence of 50% serum. Briefly, bacterial colonies from a freshly grown agar plate were re-suspended in CLSI recommended cation-adjusted Mueller Hinton broth for aerobic bacteria. MIC values were visually read at 48h. The MIC is defined as the lowest concentration producing a prominent decrease in turbidity compared to the drug free well. All assays were performed in duplicate.

The 4.6% CHD-FA solution MIC in the presence of human serum 0.125% and no serum 0.125% at 48h. Similarly, the 4.6% CHD-FA-Zn MIC in the presence of human serum is 0.03%, and no serum 0.03% at 48h.

Overall, there was no significant change in the MIC against *P. aeruginosa* treated with CHD-FA original solution and CHD-FA-Zn in the presence of human serum. (See **Table 13**.)

**Table 13. MIC of *P. aeruginosa* Xen5 to CHD-FA in the presence of human serum**

<b>P. aeruginosa Xen5</b>	<b>Type of Serum</b>	<b>MIC at 48h</b>
CHD-FA-Zn- pH 4.8	Human	0.03%
	None	0.03%
4.6% CHD-FA solution- pH 1.7	Human	0.125%
	None	0.125%

## **Section 21. Evaluation of CHD-FA zinc diacetate to treat burn wounds infected with *P. aeruginosa* strain Xen5 in 6 day study.**

To assess the efficacy of CHD-FA zinc diacetate (CHD-FA-Zn) against burn wounds infected with *P. aeruginosa* strain Xen5, 12 rats were randomized into three groups: 6 in CHD-FA-Zn (batch#2815) group, 3 in PBS group, and 3 in Silver Sulfadiazine control group. All rats were treated in 0.05ml volume at 30min post-inoculation time point on a daily basis for 6 days.

*P. aeruginosa* Xen5 was inoculated in LB media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $2 \times 10^4$  CFU per ml for the infection. Two burn wounds were created on each rat, for a total of 24 wounds. The process of anesthetizing the rats and creating the wounds was the same as previously described in burn wound infection studies. After wound creation, rats from each challenge group were infected with 0.025 ml of the *P. aeruginosa* cell suspension with final infection doses at 500 CFU/wound via injection to the burn site. Rats were given daily treatments of 0.05 ml of CHD-FA-Zn, PBS, or an adequate amount of Silver Sulfadiazine cream, starting at 30 min post inoculation. **Figure 26** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 6.

Moderate swelling of the wound beds were observed in all treatment groups from day 1 until the experimental endpoint, although the severity has subsided towards the end. Wound measurement in this infection model was not practically useful metric for treatment efficacy as the burn eschar did not shrink due to biofilm formation from overgrowth of bacteria at the wound site. Purulent discharge was observed in CHD-FA-Zn and PBS group in the early stages (up to day 3), however the duration and amount of discharge in the Silver Sulfadiazine treated rats were relatively low.

The bacterial burden assessment of the wounds at varying time points showed a minimal 0.5, 0.6, and 0.6 log burden reduction in the wounds for the CHD-FA-Zn treated group relative to the untreated controls at 24h, 48h, and 144h, respectively (**Table 14, and Figure 25**). Therefore, no significant burden reduction was observed from CHD-FA-Zn treated wounds compared to the negative control

treated wounds. Nevertheless, the Silver Sulfadiazine topical cream sterilized the infected wounds in 6 days.

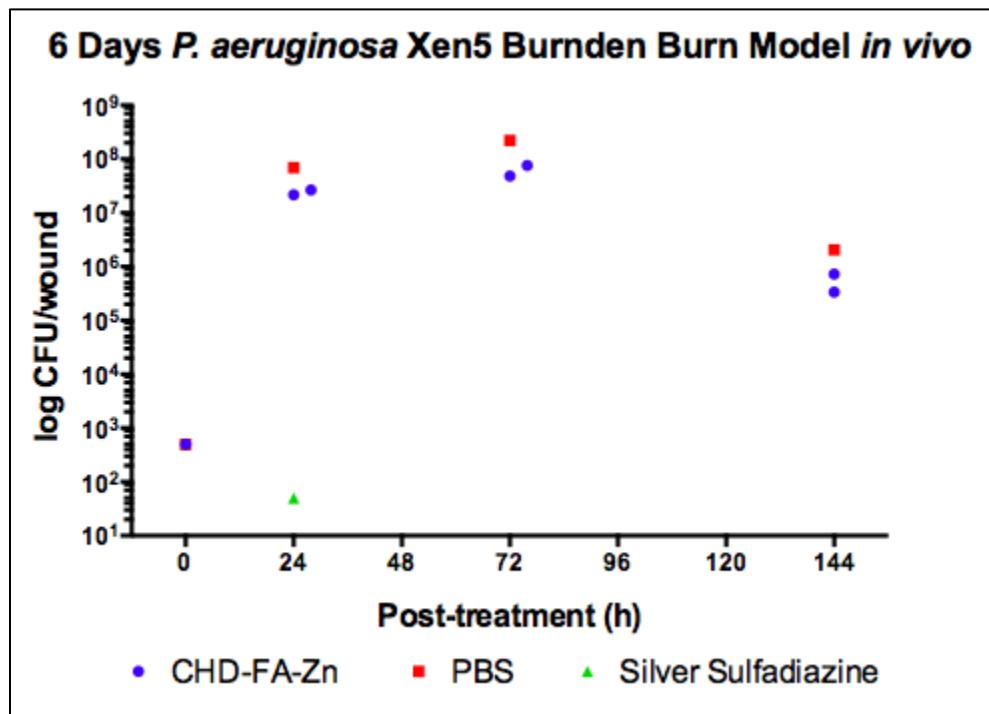
## Conclusion

Unlike the previous snapshot study, where CHD-FA-Zn sterilized the infected wounds in 2 days, CHD-FA-Zn wasn't able to keep the wounds sterile past 48 hours. The biofilm matrix formed on day 3 post-inoculation may be restricting the penetration of the drug. We will perform a snapshot study with a different application method (dressing) to enhance absorption of drugs through the burn wound eschar, and the biofilm matrix.

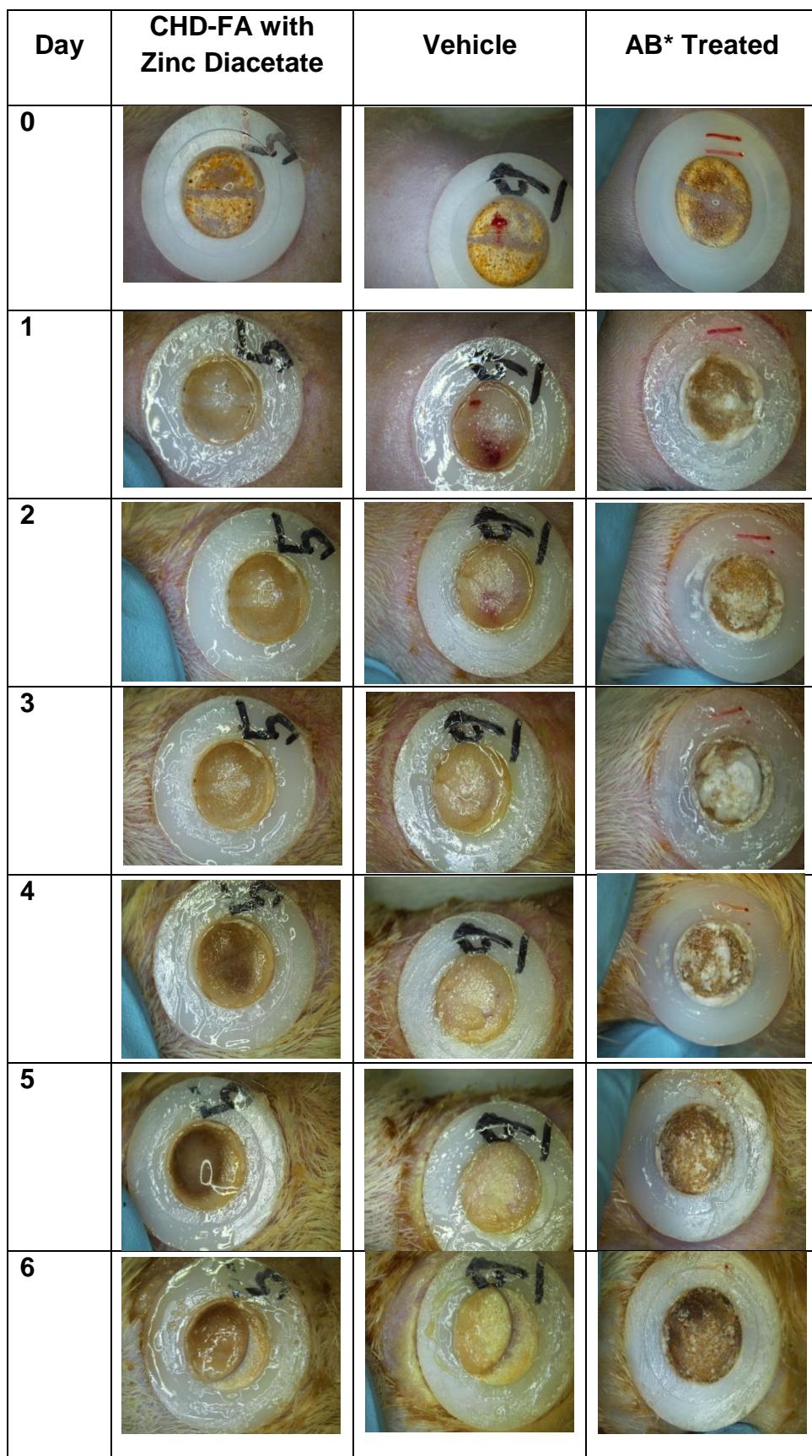
**Table 14. Bacterial burdens of burn wounds infected with *P. aeruginosa* at Day 1, 3 and 6 experimental endpoints.**

<i>P. aeruginosa</i> 500 CFU		Day 1			Day 3			Day 6			Study <i>P. aeruginosa</i> 500 CFU 0.5h Tx
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study	
No Tx Control	7.8	NA	NA	8.3	NA	NA	6.3	NA	NA		
CHD-FA wth Zinc Diacetate	7.4	7.3-7.4	0.5	7.8	7.7-7.9	0.6	5.7	5.5-5.9	0.6		
AB Control	1.7	NA	6.1	Sterilized	NA	NA	Sterilized	NA	NA		

**Figure 25. Bacterial burdens of burn wounds infected with *P. aeruginosa* vs. post-inoculation time**



**Figure 26. Burn wound images of rats infected with 500 CFU of *P. aeruginosa* (Xen5) and treated with CHD-FA zinc diacetate at 0.5h post infection.**



AB : Silver Sulfadiazine cream

## **Section 22. Evaluation of CHD-FA zinc diacetate to treat burn wounds infected with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain MW2 in 48 hr study.**

To ensure close contact of the drug with the burnt skin tissue and thus improve drug permeation through the epithelium, we have modified the application method (dressing) for treatment. A snapshot study was performed to assess the efficacy of this modified method. Four rats were randomized into two groups: 2 in CHD-FA-Zn (batch#2815) group, and 2 in PBS control group. MRSA strain MW2 was inoculated in BHI media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $2 \times 10^4$  CFU per ml for the infection. Two burn wounds were created on each rat, for a total of 8 wounds. The process of anesthetizing the rats and creating the wounds was the same as described above for the burn wound model. After wound creation, rats from each challenge group were infected with 0.025 ml of the MRSA cell suspension with final infection doses at 500 CFU/wound via intra-dermal injection to the burn site. Circular gauze dressing of 8mm in diameter was saturated overnight with either CHD-FA-Zn, or PBS in sterile centrifuge tubes. This saturated dressing of the corresponding treatments was applied to the wound site with sterile forceps twice daily starting at 30 min post inoculation. **Figure 28** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 2.

The bacterial burden assessment showed 6.7 and 6.9 log burden reduction in the wounds for the CHD-FA-Zn treated group relative to the untreated controls at 24h and 48h, respectively (**Table 15, and Figure 27**). Incorporation of drug into dressings allowed a sustained release of CHD-FA-Zn into the burn wounds and hence resulted in a rapid reduction in bacterial burden.

### **Conclusion**

In this snapshot trial, we observed that application of CHD-FA-Zn saturated dressing on the burn wound results in a rapid sterilization. Because this new application allows for greater permeation of drug into the skin, and thus keep the wounds sterile, we will use this modified method in the future burn experiments. These preliminary results are by far the most promising for the burn wound model and these studies will be repeated with a focus on the later time points to assess the bacterial burden. This new method of delivering the drug provides a strong evidence of the wound healing potentials of CHD-FA-Zn.

**Table 15. Bacterial burdens of burn wounds infected with MRSA at Day 1, and 3 experimental endpoints.**

Treatment	Day 1			Day 2			Study
	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	
No Tx Control	6.7	NA	NA	6.9	NA	NA	MRSA 500 CFU 0.5h Tx
CHD-FA wth Zinc Diacetate	Sterilized	NA	NA	Sterilized	NA	NA	

Figure 27. Bacterial burdens of burn wounds infected with MRSA vs. post-inoculation time

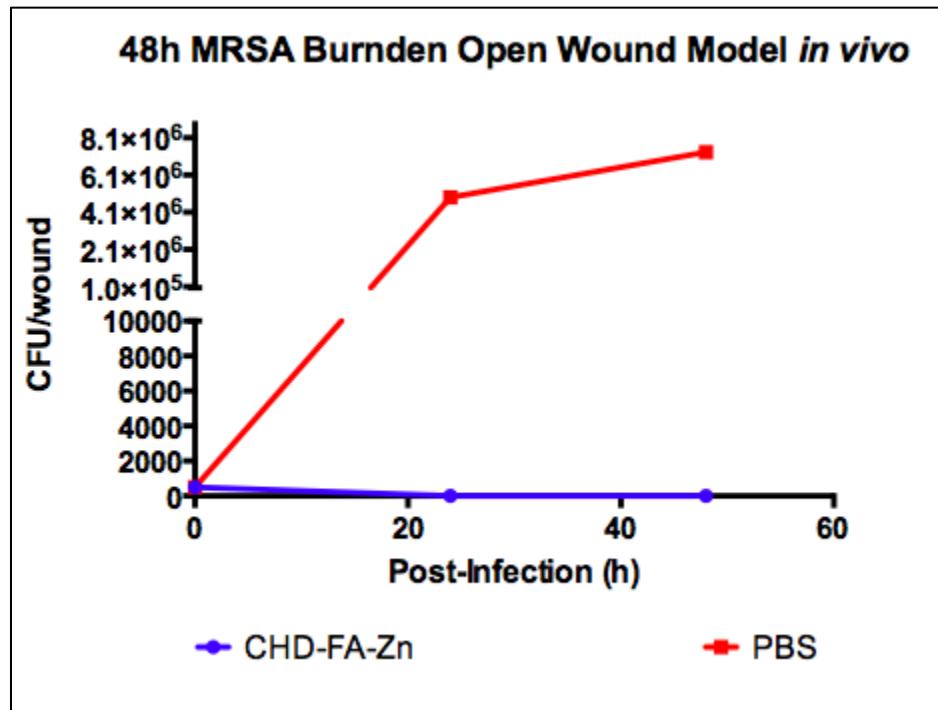
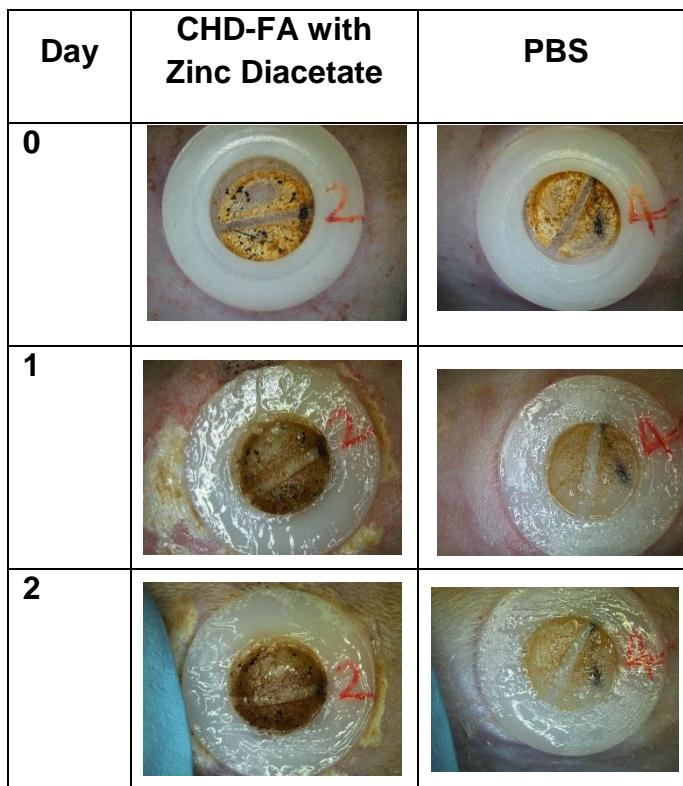


Figure 28. Burn wound images of rats infected with 500 CFU of MRSA (MW2) and treated with CHD-FA zinc diacetate at 0.5h post infection.



## **Section 23. Evaluation of CHD-FA zinc diacetate to treat cutaneous open wounds infected with *P. aeruginosa* strain Xen5 in 6 day study.**

To confirm the treatment efficacy of CHD-FA-Zn against cutaneous wounds infected with *P. aeruginosa* Xen5 in later time points, a total of 24 rats were randomized into three treatment groups: 9 in CHD-FA-Zn (batch#2815) group, 9 in Zinc Vehicle (batch#3001) group, and 6 in colistin group. All rats were treated in 0.05ml volume at 30min post-inoculation time point on a daily basis for 6 days.

*P. aeruginosa* Xen5 was inoculated in LB media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $2 \times 10^4$  CFU per ml for the infection. Two open wounds were created on each rat, for a total of 48 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the *P. aeruginosa* cell suspension to a final infection dose of 1000 CFU. Rats were given corresponding treatments in 0.05 ml volumes starting at 30min post inoculation. **Figure 30** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 6.

Moderate swelling the wound beds were observed in all treatment groups from day 1 until the experimental endpoint, although the severity has subsided towards the end. Biofilm formation on the wound surface was observed for all treatment groups from day 2 until the experimental endpoint.

The bacterial burden was sterilized for all treatment groups at 24h. However, the “sterilization” of the infection in all treatment groups was overridden by rapidly growing *P. aeruginosa* cells at 48h post-inoculation. (**Table 16, and Figure 29**). Therefore, no significant burden differences among all the groups from day 3 to day 6 experimental endpoints.

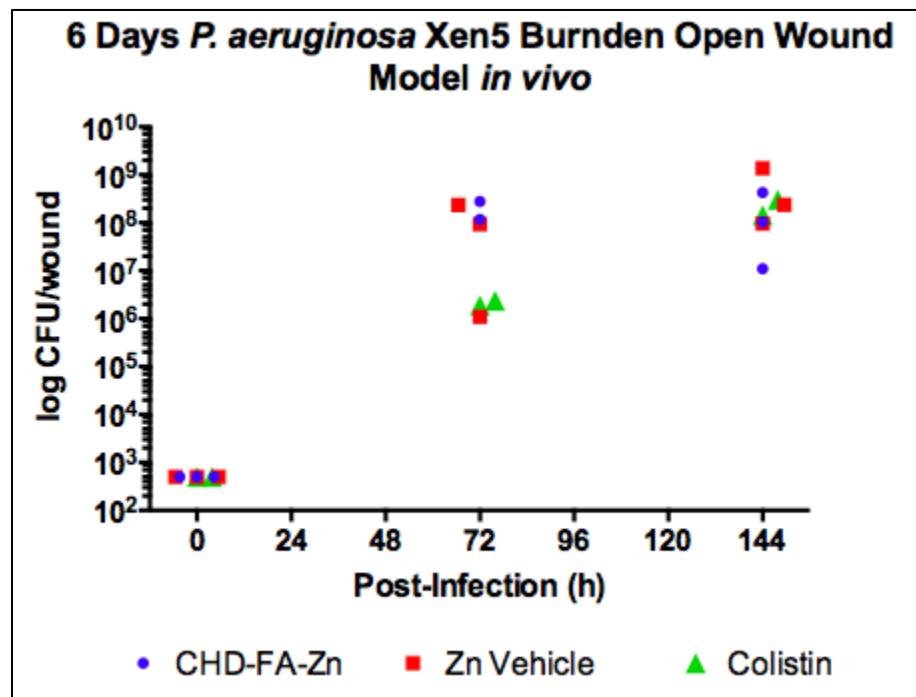
## **Conclusion**

In this 6 day study, no significant changes in microbial burden reduction were observed past 48h post-inoculation. This may be due to biofilm mixed with the scab that limit the drug absorption to the wound bed. A single application of CHD-FA-Zn was not sufficient to control infections caused by virulent and rapidly growing bacterial species such as *P. aeruginosa*. We will repeat this cutaneous open wound model infection study by using the same dressing method that we have previously proposed to use in burn model infection studies. Furthermore, we will also perform both histopathological analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA-Zn.

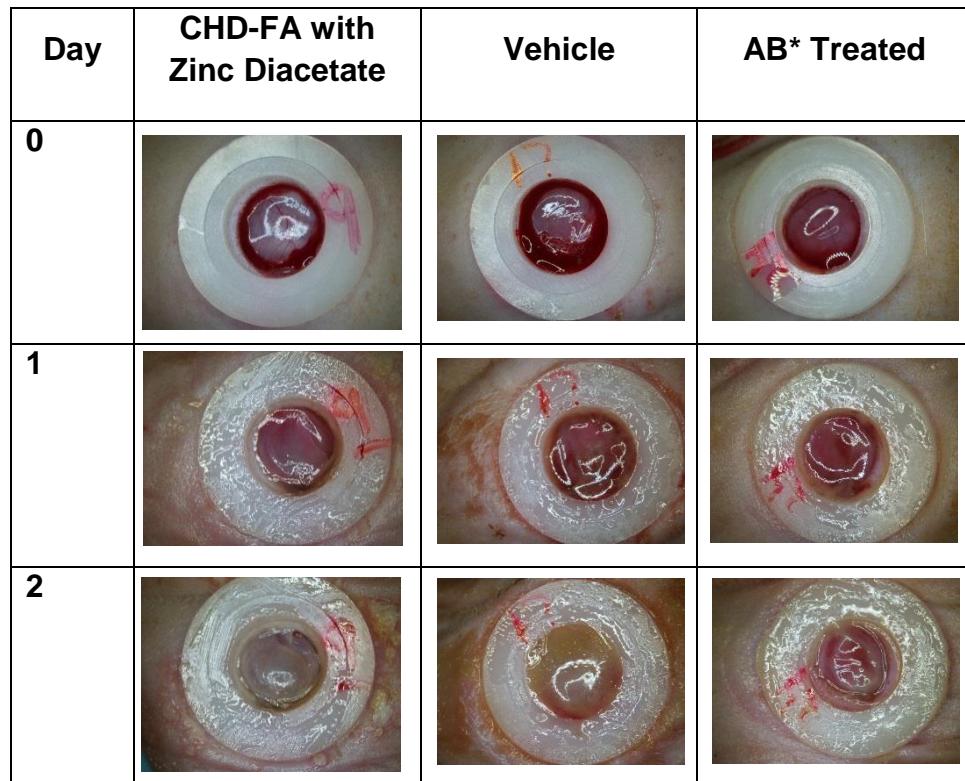
**Table 16. Bacterial burdens of open wounds infected with *P. aeruginosa* at Day 1, 3, and 6 experimental endpoints.**

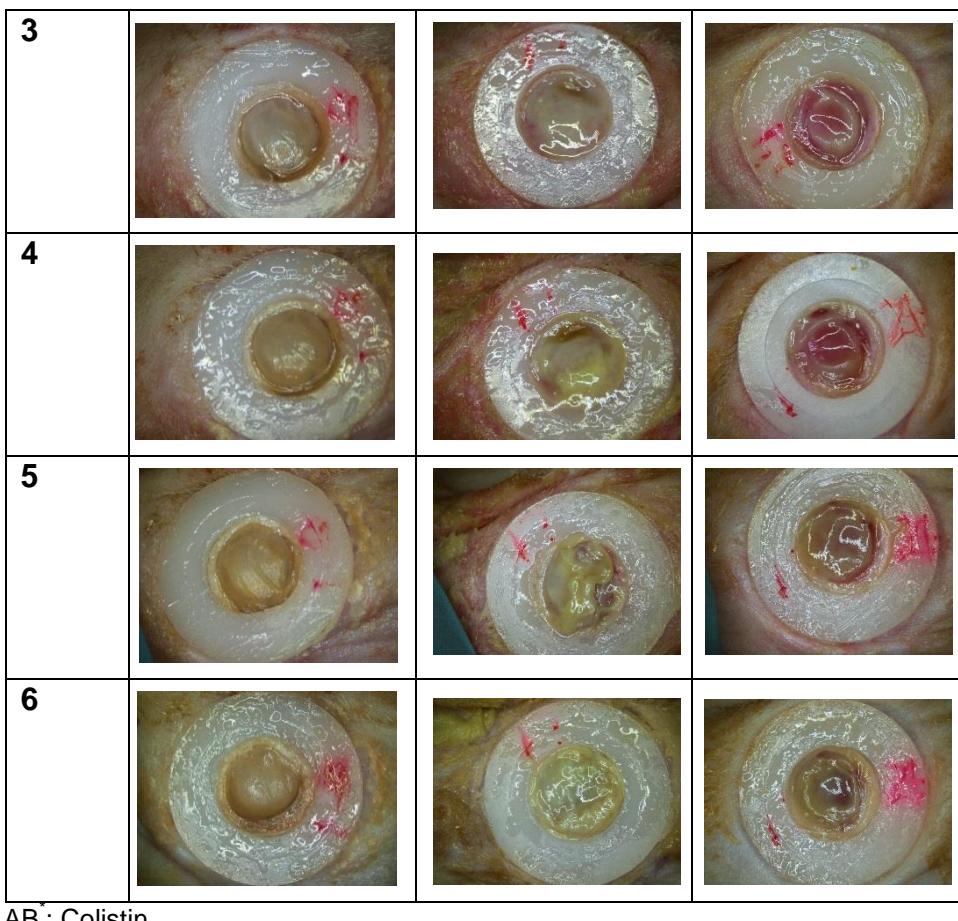
<i>P. aeruginosa</i> 500 CFU	Day 1			Day 3			Day 6			
Treatment	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Study
No Tx Control	Sterilized	NA	NA	7.5	6.0-8.4	NA	8.5	8.0-9.1	NA	<i>P. aeruginosa</i> 500 CFU 0.5h Tx
CHD-FA wth Zinc Diacetate	Sterilized	NA	NA	8.3	8.1-8.4	-0.8	7.9	7.0-8.6	0.6	
AB Control	Sterilized	NA	NA	6.3	6.3-6.4	1.1	8.3	8.2-8.5	0.2	

**Figure 29. Bacterial burdens of open wounds infected with *P. aeruginosa* vs. post-inoculation time**



**Figure 30. Cutaneous open wound images of rats infected with 1000 CFU of *P. aeruginosa* (Xen5) and treated with CHD-FA zinc diacetate at 0.5h post infection.**





AB : Colistin

#### **Section 24. Evaluation of CHD-FA zinc diacetate to treat cutaneous open wounds infected with Methicillin Resistant *Staphylococcus aureus* (MRSA) strain MW2 in 6 day study.**

A low dose MRSA cutaneous wound infection was performed to address the microbial burden persistency observed in the previous *P. aeruginosa* infection model with CHD-FA-Zn. A total of 24 rats were randomized into five treatment cohorts: CHD-FA zinc diacetate gel (batch#2815), 2% mupirocin cream, and PBS control. All treatments were initiated at 30min post-inoculation and applied on a daily basis.

Methicillin Resistant *S. aureus* strain MW2 was inoculated in Brain Heart Infusion (BHI) media and incubated at 37°C with shaking overnight. Bacterial cells were washed, precipitated in sterile PBS, and diluted to  $1 \times 10^4$  CFU/ml. Two open wounds were created on each rat, for a total of 48 wounds. The process of anesthetizing the rats and creating the open wounds was the same as previously described in cutaneous wound infection studies. After wound creation, rats from each treatment group were infected with 0.05 ml of the MRSA cell suspension to a final infection dose of 500 CFU. Rats were given corresponding treatments in 0.05 ml volumes starting at 30min post inoculation. The 30min treatment starting time was used to determine if significant improvement in burden reduction would be observed. **Figure 32** shows representative images of the wounds over the course of day 0 (day of burn wound creation) through day 6. The bacterial burden assessment of the wounds at varying time points showed a 4.1 log burden reduction in the wounds for the CHD-FA-Zn treated group relative to the untreated controls at 24h. However, recovery of the microbial burden was observed in CHD-FA-Zn treated group past 48h. (**Table 17, and Figure 31**). Therefore, no significant burden reduction was observed from CHD-FA-Zn treated wounds compared to the negative control

treated wounds from day 3 to day 6 experimental endpoints. Nevertheless, the mupirocin topical cream kept the wound sterilized throughout the experiment.

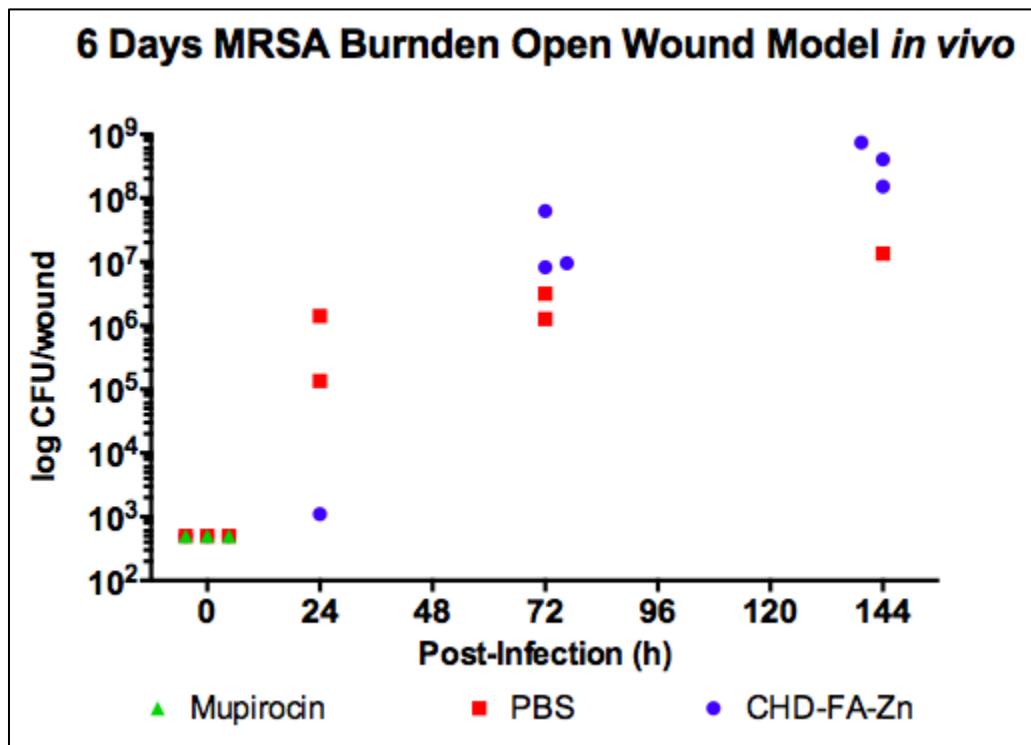
## Conclusion

As seen with the previous 6 day infection study with *P. aeruginosa*, no significant changes in microbial burden reduction were observed past 48h post-inoculation. This may be due to biofilm mixed with the scab that limit the drug absorption to the wound bed. A single application of CHD-FA-Zn was not sufficient to control infections caused by this bacterial species. We will repeat this cutaneous open wound model infection study by using the same dressing method that we have previously used in burn infection 48h snapshot studies. Furthermore, we will also perform both histopathological analyses and host gene expression profiling to better assess the cellular and molecular mechanisms during wound healing with CHD-FA-Zn.

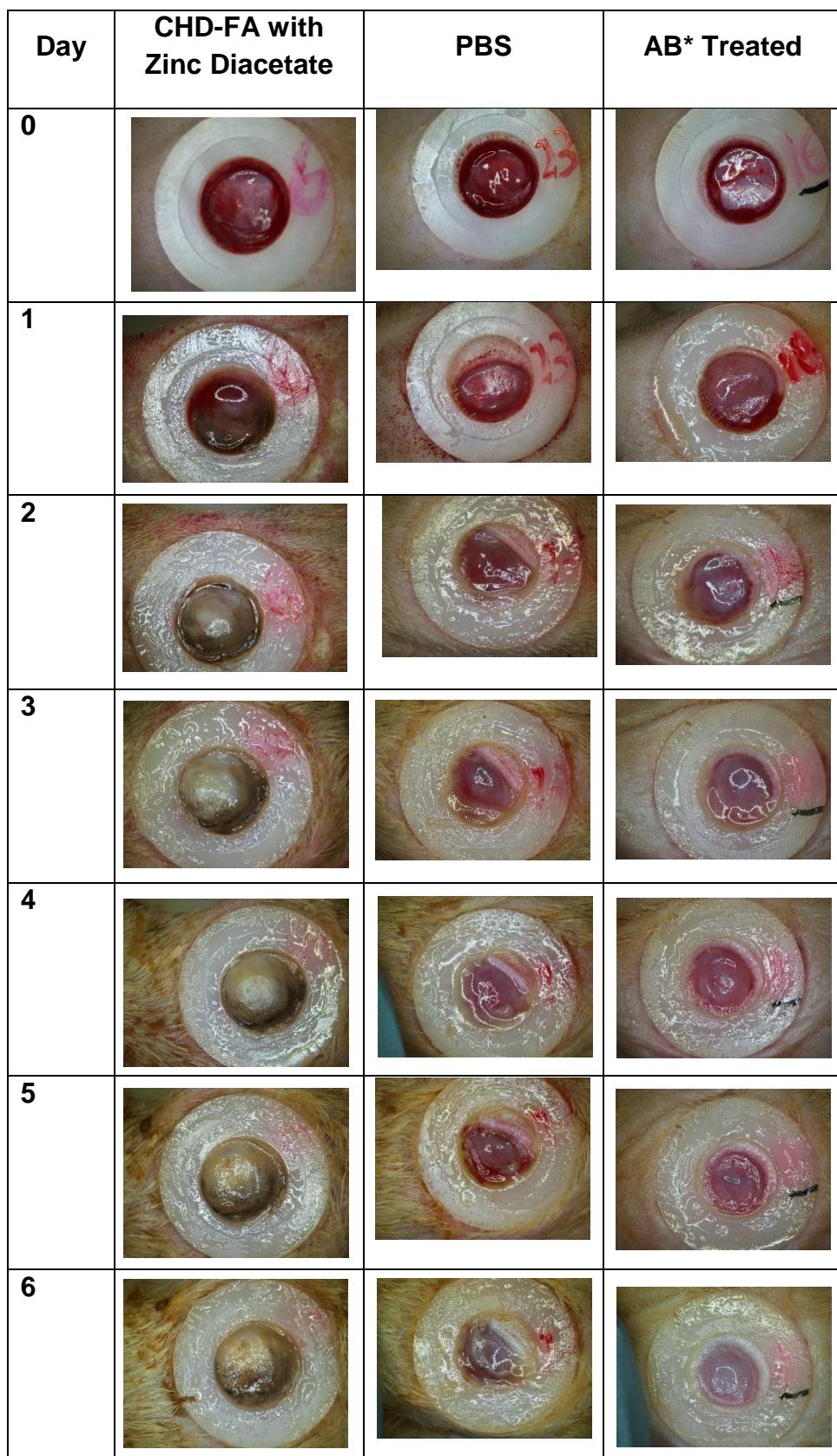
**Table 17. Bacterial burdens of open wounds infected with MRSA at Day 1, 3, and 6 experimental endpoints.**

Treatment	Day 1			Day 3			Day 6			Study
	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	Avg. Log CFU	Range	Log Fold Changes	
No Tx Control	5.6	5.1-6.1	NA	6.3	6.1-6.5	NA	7.1	NA	NA	
CHD-FA wth Zinc Diacetate	1.5	0.0-3.0	4.1	7.2	6.9-7.8	-0.9	8.6	8.2-8.9	-1.4	
AB Control	Sterilized	NA	NA	Sterilized	NA	NA	Sterilized	NA	NA	

**Figure 31. Bacterial burdens of open wounds infected with MRSA vs. post-inoculation time**



**Figure 32. Cutaneous open wound images of rats infected with 500 CFU of MRSA (MW2) and treated with CHD-FA zinc diacetate at 0.5h post infection.**



AB : 2% mupirocin cream

## Key Research Accomplishments

- Demonstrated that bactericidal properties of CHD-FA are dependent on pH both *in vitro* and *in vivo* and modified the formulation of CHD-FA to stabilize the pH
- Established a reproducible burn wound model in rats for bacteria that can be used to assess CHD-FA to treat burn wound infections
- Obtained histopathological analyses of burn wounds from rats infected with *Klebsiella pneumoniae*, Methicillin Resistant *Staphylococcus aureus*, and treated with CHD-FA
- Performed host gene expression profiling of *Klebsiella pneumoniae*, Methicillin Resistant *Staphylococcus aureus*, infected burn wounds, and demonstrated that CHD-FA-Zn blocks cellular inflammation and improves wound healing
- Obtained preliminary efficacy data on CHD-FA-Zn to treat *Pseudomonas aeruginosa*, and Methicillin Resistant *Staphylococcus aureus* cutaneous and burn wound infections in rats.
- Our initial results demonstrate CHD-FA-Zn significantly reduced microbial burdens compared to the untreated control groups in 48h burn wound infected with MRSA, and *Pseudomonas aeruginosa*

## **Reportable Outcomes**

In addition to the previous poster presentation (**CHD-FA is a highly effective topical broad-spectrum antimicrobial for drug-resistant wound infections**) at the 54<sup>th</sup> Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC 2014, Washington DC) and the oral presentation entitled **“Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections”** at the Military Health System Research Symposium (MHSRS) 2014, we have published an original article “Carbohydrate-derived fulvic acid is a highly promising topical agent to enhance healing of wounds infected with drug-resistant pathogens” on the Journal of Trauma and Acute Care Surgery in October 2015. During the past year, we have successfully established and optimized the burn wound model. To resolve the problem of largely diminished *in vivo* antimicrobial activities of the original CHD-FA formulation, we have made great efforts in prompting CHD-FA formulation modification as well as in evaluating new formulations comprehensively and critically. By far, the CHD-FA-Zn is the most promising formulation, which demonstrated a significantly increased antimicrobial activities in the short-course studies on both cutaneous and burn wounds infected with MRSA.

Dr. Perlin was awarded a NIH Research Program – Cooperative Agreements (U19) grant (RFA-AI-12-044) that resulted in PHRI-Rutgers University being designated as one of the NIAID Centers for Excellence in Translational Research (CETR) to develop novel leads against drug resistant pathogens. The wound model was a significant component in the assessment of novel lead compounds against drug resistant bacterial pathogens.

## Conclusion

In this third annual report, a burn wound model in rats was established and infection with *K. pneumoniae*, MRSA, *P. aeruginosa* and treated with CHD-FA were validated. Histopathological and host gene expression profiling analyses of burn wounds infected with *K. pneumoniae* and MRSA and treated with CHD-FA demonstrated improved healing at the cellular and molecular level. Specifically, CHD-FA treated wounds displayed decreased cellular inflammation, and increased rates of epithelialization.

We have performed the *in vivo* analysis of the newly formulated CHD-FA-Zn. Our initial results demonstrate that CHD-FA-Zn can reduce microbial burdens of susceptible and drug-resistant planktonic bacteria, and significantly improved wound healing kinetics. Host expression profiling of wound healing genes showed similar behavior with CHD-FA treated wounds with a prominent effect of reducing inflammation at early stages of infection. Our studies have raised some open questions. For example, cutaneous wounds infected with MRSA and *P. aeruginosa*, and treated with CHD-FA-Zn had rapid microbial clearance until 48h post-inoculation. However, the microbial burdens restored from day 3 till day 6. The biofilm matrix formed on day 3 post-inoculation may be restricting the penetration of the drug. During the next quarter and throughout next year, we will perform studies with a modified application method (dressing) to enhance absorption of drugs through the burn wound eschar and the biofilm matrix. And once the efficacy of the modified method with CHD-FA-Zn is determined, we will perform additional studies with other pathogens listed in the statement of work. Overall, we have made strong progress in the third year. We believe that the newly formulated CHD-FA combined with the modified application method will be effective in treating wounds infected with clinically important drug resistant pathogens and demonstrate its universality for preventing wound infections and promote healing.

## References

1. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. CXC chemokines in angiogenesis. *Journal of leukocyte biology.* 2000;68(1):1-8.
2. Orman MA, Nguyen TT, Ierapetritou MG, Berthiaume F, Androulakis IP. Comparison of the cytokine and chemokine dynamics of the early inflammatory response in models of burn injury and infection. *Cytokine.* 2011;55(3):362-71.
3. Bussolino F, Wang JM, Defilippi P, Turrini F, Sanavio F, Edgell CJ, Aglietta M, Arese P, Mantovani A. Granulocyte- and granulocyte-macrophage-colony stimulating factors induce human endothelial cells to migrate and proliferate. *Nature.* 1989;337(6206):471-3.
4. Shah JM, Omar E, Pai DR, Sood S. Cellular events and biomarkers of wound healing. *Indian journal of plastic surgery : official publication of the Association of Plastic Surgeons of India.* 2012;45(2):220-8.
5. Chen L, Arbieva ZH, Guo S, Marucha PT, Mustoe TA, DiPietro LA. Positional differences in the wound transcriptome of skin and oral mucosa. *BMC genomics.* 2010;11:471.
6. Ladwig GP, Robson MC, Liu R, Kuhn MA, Muir DF, Schultz GS. Ratios of activated matrix metalloproteinase-9 to tissue inhibitor of matrix metalloproteinase-1 in wound fluids are inversely correlated with healing of pressure ulcers. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society.* 2002;10(1):26-37.
7. Norgauer J, Hildenbrand T, Idzko M, Panther E, Bandemir E, Hartmann M, Vanscheidt W, Herouy Y. Elevated expression of extracellular matrix metalloproteinase inducer (CD147) and membrane-type matrix metalloproteinases in venous leg ulcers. *The British journal of dermatology.* 2002; 147(6):1180-6.
8. Hasegawa M, Higashi K, Matsushita T, Hamaguchi Y, Saito K, Fujimoto M, Takehara K. 2013. Dermokine inhibits ELR(+)CXC chemokine expression and delays early skin wound healing. *Journal of dermatological science* 70:34-41.
9. Simonetti, O., et al., Tigecycline accelerates staphylococcal-infected burn wound healing through matrix metalloproteinase-9 modulation. *J Antimicrob Chemother.* 2012. 67(1): p. 191-201.
10. Jang, J., et al., Wound healing effect of cuttlebone extract in burn injury of rat. *Food Science and Biotechnology,* 2013. 22(1): p. 99-105.

## Appendices

### Updated Quad Chart

#### Evaluation of Carbohydrate Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug Resistant Wound Infections



Insert ERMS/Log Number and Task Title Here

W81XWH-11-DMRDP-MID-ARA

PI: David S. Perlin Org: New Jersey Medical School-Rutgers, The State University of New Jersey Award Amount: \$1,530,400

#### Study/Product Aim(s)

- Assess *in vitro* susceptibility of CHD-FA against multi-drug resistant bacteria and fungal pathogens
- Evaluate efficacy of topical application of CHD-FA on small animal models of wound infection with a wide variety of bacterial and fungal pathogens.

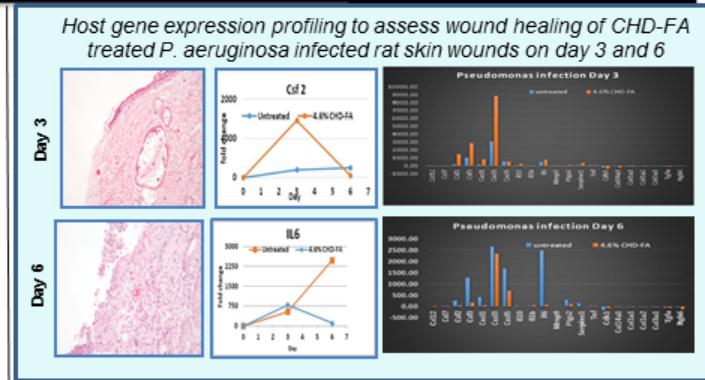
#### Approach

Establish minimum inhibitory concentrations ( $MIC_{50}$  and  $MIC_{90}$ ) for CHD-FA against large collections of clinical isolates representing wound-associated drug resistant bacteria and fungi. Rat models of wound infection (open, burn and deep tissue) will be established with healthy animals using drug resistant bacterial and fungal pathogens. Increasing doses of CHD-FA in topical form will be used to assess relative efficacy.

#### Timeline and Cost

Activities	CY 12	13	14	15
Establish $MIC_{50}$ s for CHD-FA with clinical isolates of major drug resistant pathogens				
Assess CHD-FA in animal models of wound infection for major pathogens				
<i>in vivo</i> evaluation of CHD-FA in open, burn and deep tissue models.				
Histopathological analysis and gene expression profiling.				
<b>Estimated Budget (\$K)</b>	\$400	\$600	\$500	\$400

Updated: Oct 29, 2015



#### Goals/Milestones

- CY12 Goal – Establish ( $MIC_{50}$  and  $MIC_{90}$ ) for CHD-FA
- in vitro* efficacy of CHD-FA against drug resistant bacteria.
- CY13 Goals – Complete *in vitro* susceptibility testing and establish cutaneous wound model in rats.
- demonstrated the potent anti-microbial activity of CHD-FA against a broad range of drug resistant bacteria and fungi.
- CY14 Goal – Establish open/ burn wound infection model in rats using drug resistant bacterial and fungal pathogens following treatment with CHD-FA in topical form.
- established the cutaneous wound model in rats with major pathogens.
- CY15 Goal – *in vivo* evaluation of CHD-FA in deep tissue models in rats.
- established the burn wound model in rats with MRSA, *P. aeruginosa* & *K. pneumoniae* following treatment with CHD-FA-Zn in topical form.
- histopathological analyses and host gene expression profiling to assess burn wound healing.
- Comments/Challenges/Issues/Concerns**
- It remains to be determined if a single application of CHD-FA-Zn is sufficient to control the infection long term. We will also extend the pathogen spectrum for this evaluation.

#### Budget Expenditure to Date

Projected Expenditure:\$1,064,094

Actual Expenditure:\$1,513,482

**“Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections.”**

***Quarterly Technical Progress Report (Q9.01.2015)***

**Performance period:** 09/30/2014 through 12/31/2014.

**Date of report:** January 15, 2015

**Public Health Research Institute  
New Jersey Medical School, Rutgers, The State University of New Jersey  
225 Warren Street  
Newark, NJ 07103**

**Award Recipient/Principal Investigator:**

**David S. Perlin, Ph.D.  
Tel. 973-854-3200**

**Grants Officer:**

**Alla Rabinovich, M.B.A.  
Tel. 973-854-3115**

**Quarterly technical report Q10.04.2015**

**“Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections.”**

***Quarterly Technical Progress Report (Q10.04.2015)***

**Performance period: 12/31/2014 through 3/31/2015.**

**Date of report: April 15, 2015**

**Public Health Research Institute  
New Jersey Medical School, Rutgers, The State University of New Jersey  
225 Warren Street  
Newark, NJ 07103**

**Award Recipient/Principal Investigator:**

**David S. Perlin, Ph.D.  
Tel. 973-854-3200**

**Grants Officer:**

**Alla Rabinovich, M.B.A.  
Tel. 973-854-3115**

**Quarterly technical report Q11.07.2015**

**“Evaluation of Carbohydrate-Derived Fulvic Acid (CHD-FA) as a Topical Broad-Spectrum Antimicrobial for Drug-Resistant Wound Infections.”**

***Quarterly Technical Progress Report (Q11.07.2015)***

**Performance period: 4/1/2015 through 7/1/2015.**

**Date of report: July 15, 2015**

**Public Health Research Institute  
New Jersey Medical School, Rutgers, The State University of New Jersey  
225 Warren Street  
Newark, NJ 07103**

**Award Recipient/Principal Investigator:**

**David S. Perlin, Ph.D.  
Tel. 973-854-3200**

**Grants Officer:**

**Alla Rabinovich, M.B.A.  
Tel. 973-854-3115**